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## ON THE FORSSMAN ANTIGENS IN *B. PARATYPHOSUS* B AND *B. DYSENTERIAE* SHIGA

BY K. LANDSTEINER AND PHILIP LEVINE

(From the Laboratories of The Rockefeller Institute for Medical Research)

The occurrence of substances in bacilli of the *Salmonella* group,<sup>1</sup> inducing in rabbits the formation of sheep hemolysins, so-called Forssman antibodies, has been described by various authors (4), (5), (6), (7).<sup>2</sup> Such antigens have been found by Seiffert (6) in strains of *B. paratyphosus* (Schottmüller and Freiburg) and of *B. enteritidis* Gaertner, and by Meyer in the types Newport and Berlin. Other bacilli which have been shown to contain Forssman antigens, are *B. dysenteriae* Shiga (9), (10), a particular strain of *B. leptisepticus* (11), and pneumococci (12), (13).

That the various bacillary antigens are not identical, has been shown by Jungeblut and Ross for *B. paratyphosus* B and *B. dysenteriae* Shiga, and Meyer has demonstrated that immune sera containing sheep lysin, produced by four different strains of the *Salmonella* group (*B. paratyphosus* B, B Berlin, B Newport, and *B. enteritidis* Gaertner) were absorbable only by the homologous organisms (see Eisler (14)).

It has been pointed out that the Forssman antigen in *B. paratyphosus* B or *B. dysenteriae* Shiga is not identical with the Forssman antigen present in sheep blood and in the organs of guinea pigs (15), (10), (5), (6), cf. (9). One sort of microorganism, namely *B. leptisepticus* was found by Powell and by ourselves to absorb<sup>3</sup>

<sup>1</sup> With regard to the terminology and the classification of the various strains of the *Salmonella* group and the factors assigned to the bacilli reference may be made to the paper by Bruce White (1), (2), and Kaufmann (3).

<sup>2</sup> On the occurrence in anti-paratyphosus B sera of agglutinins for human blood of group A see (8).

<sup>3</sup> In this connection it is worth mentioning that also in the immunization *B. leptisepticus* shows a high activity inasmuch as in our experience it is very easy to produce hemolytic immune sera by the use of these bacilli.



Forssman antibodies such as are obtained by the injection of guinea pig organs or boiled sheep blood, and more recently such an observation has been reported by Bailey and Shorb for pneumococcus type I.

In the following some observations are recorded concerning the occurrence and properties of the Forssman antigen in *B. Aertryck*<sup>4</sup> and *B. dysenteriae* Shiga.

#### EXPERIMENTAL

The hemolytic serum used in the present tests was prepared by three intravenous injections, made at intervals of five days, of a heated bacillary suspension of several different strains of *B. dysenteriae* Shiga. The amount used for each injection was equivalent to one-hundredth of a slant. The hemolytic Aertryck immune sera were prepared by weekly injections of bacilli heated to 60° for thirty minutes, in quantities equivalent to one-twentieth to one-tenth of a slant.

The tests for hemolysis were made as given previously (17).

#### DIFFERENCES IN THE FORSSMAN ANTIGENS OF VARIOUS BACILLI

Experiments on the absorption of hemolysins contained in antibacterial sera gave results which were in agreement with the findings quoted above. The hemolysins formed after the injection of three different sorts of bacilli, namely *B. Aertryck*, *B. dysenteriae* Shiga, and *B. leptisepticus*, proved to be different, since the first two bacilli absorbed the lysins only from the corresponding immune sera and not from the *B. leptisepticus* immune serum which, in turn, was absorbable by the homologous bacilli. A part of the data supporting this statement, is contained in table 1.

From the survey of the literature and the observations on sheep hemolysins produced by various animal cells (see (18)), it appears that the capacity of hemolyzing sheep blood is a property of a large number of otherwise different antibodies and that the corresponding so-called Forssman antigens are not identical but have some sort of similarity. It does not seem reasonable to

<sup>4</sup> The Salmonella cultures used were the same as those studied previously (16) and were obtained from the National Collection of Type Cultures, Lister Institute, London.

suppose that sheep blood which is acted upon by a number of immune sera contains an equivalent number of groupings each of them related to one of these hemolysins, and it is a much more natural assumption that sheep blood contains one or perhaps several Forssman substances possessing the property of reacting with various antibodies.

DISTRIBUTION OF THE FORSSMAN ANTIGEN CONTAINED IN  
B. AERTRYCK

As mentioned above, investigations concerning the occurrence of Forssman antigens in different bacilli of the Salmonella group have been made by K. Meyer. In our experiments with an anti-Aertryck immune serum, a number of strains were tested and the results are presented in table 1.

The inhibition of the hemolytic action of the anti-Aertryck immune sera by the bacillary suspension of *B. paratyphosus* Dawes is probably due to an anti-complementary action inasmuch as a similar effect was not noted in the tests with the bacillary extracts. These bacilli inhibited also the lytic action of an anti-horse kidney immune serum.

From the tests tabulated, there appears a certain regularity in the absorption of the hemolysin by the bacillary suspensions as well as by the bacterial extracts. In terms of the agglutinable factors assigned to the various bacilli by Bruce White, only the strains containing the factor I absorbed the hemolysin while the others did not act under the same conditions. Particularly three strains, namely, *B. abortus equinus*, *B. Derby*, and *B. Reading*, which have factor II in common with the absorbing strains, were devoid of binding capacity. The same result was obtained with the use of another anti-Aertryck immune serum. Hence, the Forssman antigen in question appears to run parallel with factor I. To give an explanation for the differences between these results and those obtained by K. Meyer would require further investigation; the discrepancy perhaps may be due to variations in the properties of the immune sera.

In spite of the relationship observed, the precipitins and agglutinins for factor I on the one hand, and the hemolytic antibodies

TABLE 1

A. The bacterial contents of a twenty-four-hour agar slant were taken up in saline and centrifuged after previous heating at 60° for thirty minutes. The bacteria were resuspended in 2 cc. of saline. To 0.5 cc. of 2 hemolytic units of anti-Aertryck immune serum was added 0.1 cc. of the given dilutions of the various bacterial suspensions. After fifteen minutes fixation at 37°C., 0.5 cc. of complement diluted 1:15 and one drop of a 50 per cent suspension of washed sheep blood were added. Readings were made at the end of fifteen minutes incubation at 37°C.

B. The bacterial contents of one slant were taken up in 4 cc. of saline, heated at 60° for thirty minutes, and then centrifuged. Five-tenths cubic centimeter of the clear supernatant bacterial extracts, diluted as stated, were mixed with 0.5 cc. of two hemolytic units of anti-Aertryck immune serum and kept for one hour at 37°C. at the end of which interval 0.5 cc. of complement diluted 1:10 and one drop of a 50 per cent suspension of sheep blood were added. The readings were made at the end of fifteen minutes' incubation at 37°C.

The strength of the hemolytic reactions is indicated as follows: 0 = no hemolysis, tr. = trace, d. = distinct, a.c. = almost complete, c. = complete.

	HEAT STABLE FACTORS (WHITE)	A. TESTS WITH BACTERIAL SUS- PENSION DILUTED		B. TESTS WITH BACTERIAL EX- TRACT DILUTED	
		1:5	1:20	1:5	1:20
Aertryck.....	I, II		0	0	0
Paratyphosus B, strain 1.....	I, II		0	0	0
Paratyphosus B, strain 2 (Cools).....	I, II		0	0	0
Paratyphosus B, strain 3 (Tidy).....	I, II		0	0	0
Stanley.....	I, II		0	0	0
Abortus equinus.....	II	c.	c.	c.	
Derby.....	II	c.	c.	a.c.	
Reading.....	II	c.	c.	a.c.	
Enteritidis.....	III	c.	c.	c.	
Typhosus.....	III	c.	c.	c.	
Glasser-Voldagsen.....	V	c.	c.	c.	
Newport.....	V, VI	c.	c.	c.	
American suipestifer.....	V, VI	c.	c.	c.	
Paratyphosus A, strain Rogers.....	XI	c.	c.	a.c.	
Paratyphosus A, strain Dawes.....	XI	tr.	a.c.	c.	
B. dysenteriae, Shiga.....		c.	c.	c.	

on the other, are not identical. In the first place, anti-Aertryck immune sera which contain precipitins and agglutinins active for factor I, need not contain sheep hemolysin. Secondly, when solutions were prepared by complete absorption of anti-Aertryck immune serum, with *e. g.*, *B. abortus equinus*, it was possible to

absorb the hemolysins with sheep blood, without diminishing the agglutination titer for bacilli containing factor I. That the absorption of the hemolysin for sheep blood does not remove the bacterial agglutinins and precipitins, has been shown in other similar cases by Jungeblut and Ross, and K. Meyer.

Tests made with two strains of *B. Binns*,<sup>5</sup> supposed to contain factor I, showed that neither strain absorbed the anti-Aertryck lysin. However, these bacilli were not distinctly agglutinated by an absorbed fluid specific for factor I.

#### ACTION OF ACID AND ALKALI ON THE FORSSMAN ANTIGENS IN *B. AERTRYCK* AND *B. DYSENTERIAE* SHIGA

On examining the action of acid on the bacterial extracts<sup>6</sup> it was found that the lysin-binding capacity was diminished after five minutes heating in the steam bath in *N*/10 solution of hydrochloric acid and was practically destroyed after the solution had been heated for an hour; the rate of decrease in activity was about the same for *B. Aertryck* and *B. dysenteriae* Shiga. In precipitin tests similar results were obtained.

In the resistance of the two extracts to alkali, there was a distinct difference. The activity of the Shiga extract was diminished after thirty minutes heating in *N*/1 solution of sodium hydroxide, but remained constant on further heating for additional ninety minutes. In contrast to this, the Aertryck substance proved to be sensitive to the action of alkali, so that treatment with *N*/10 sodium hydroxide for thirty minutes at room temperature was sufficient to destroy the activity almost completely. A slight activity, which persisted even after heating for thirty minutes in *N*/10 sodium hydroxide, is probably due to an anti-complementary effect. Heating with 0.5 per cent sodium carbonate for thirty minutes also destroyed the binding power.

The observations described gain significance in view of previous

<sup>5</sup> We are indebted to the National Collection of Type Cultures for supplying us with these cultures.

<sup>6</sup> These substances were made according to the method of extraction with saline solution described by Furth and Landsteiner (Jour. Exp. Med., 1929, xlix, 729) except that no alkali was used.

TABLE 2

To a 1 per cent solution of crude substance was added sodium hydroxide or hydrochloric acid in the concentrations indicated below. After the intervals stated, the mixtures were neutralized and tested for their anti-hemolytic and their precipitating properties (the precipitin tests are not tabulated).

The anti-hemolytic tests were made as follows: To 0.5 cc. of 2 hemolytic units were added 0.5 cc. dilution of the various mixtures and incubated for thirty minutes at 37°, when 0.5 cc. guinea pig serum 1:10 and one drop of 50 per cent washed sheep blood were added.

		DILUTION OF THE EXTRACT					
		1:10 <sup>6</sup>	1:10 <sup>4</sup>	1:10 <sup>3</sup>	1:10 <sup>2</sup>	1:10 <sup>1</sup>	1:10 <sup>0</sup>
Tests for anti-hemolytic action with crude extract of <i>B. Aertryck</i> and Aertryck hemolytic immune serum							
Aertryck extract treated with:							
N/10 NaOH.....	30 minutes at room temperature	d.	c.				
N/10 NaOH.....	30 minutes at 100°	tr.	c.				
N/10 HCl.....	5 minutes at 100°	—	0	0	c.		
N/10 HCl.....	1 hour at 100°	tr.	c.				
Not treated.....				0	0	c.	c.

Anti-hemolytic tests with crude extract of *B. dysenteriae* Shiga and Shiga hemolytic immune serum

Shiga extract treated with:							
N/1 NaOH.....	30 minutes at 100°	—	—	0	0	d.	c.
N/1 NaOH.....	2 hours at 100°	—	0	0	0	d.	c.
N/10 HCl.....	5 minutes at 100°	—	0	0	0	c.	
N/10 HCl.....	1 hour at 100°	tr.	c.				
Not treated.....		—	—	0	0	0	d. c.

On heating the alkaline mixture slight precipitates were formed which disappeared on neutralization. A precipitation occurred also on boiling with N/10 hydrochloric acid. The anti-hemolytic tests were made without removing the insoluble substance.

similar results concerning the action of alkali on the precipitable substance of *B. paratyphosus* B (16). In these experiments, precipitin reactions for factor I made with the aid of an anti-paratyphosus B serum absorbed with *B. Reading* occurred only when alkali was avoided in the preparation of the extracts of *B. paratyphosus* B. A precipitable substance which was made by using

trichloroacetic acid for the removal of proteins was found to be destroyed after treatment with weak solutions of sodium hydroxide or sodium carbonate.

In our experiments, the precipitin reactions also were diminished considerably after heating for thirty minutes in N/10 sodium hydroxide; the remaining activity is presumably attributable to the alkali-stable factor II.

If one puts together the fact that in our experiments the special Forssman substance was found only in strains containing factor I and, secondly, the alkali lability of both the precipitable factor I and the Forssman antigen, a mere coincidence would seem less likely than a close connection between the two functions which could depend on one substance, perhaps with two distinct groupings. If this were the case, it would follow that one antigenic substance can cause the formation of different antibodies, a view implied in the pluralistic hypothesis on the action of immune sera and discussed particularly by K. Meyer for the hemolytic anti-bacterial sera.

The recent studies on the properties of Forssman antigens in bacilli and their relation to the heat stable agglutinin are suggestive of a carbohydrate nature (19), (20), (14), and a similar view has been pointed out also for the specific groupings of the Forssman hapten in animal tissues (21). This offers an explanation for the group reactions of a number of different substances inasmuch as it has been shown (Avery, Heidelberger and Goebel) that various specific carbohydrates may contain the same sugars and sugar acids as chief constituents. In order to obtain conclusive evidence, investigations on purified material seems necessary.

*Addendum:* After the completion of this paper, a communication by Eisler (Z. f. Immunitätsf. 1931, 73, 37) appeared, dealing with the action on blood of *B. paratyphosus* B and *B. dysenteriae* Shiga immune sera in which he describes antihemolytic activity of alcoholic extracts of *B. paratyphosus* B and an action of anti-paratyphosus B serum on human blood of group A. In Eisler's experiments also two strains of *B. Gaertner* were capable of binding the hemolysin produced by *B. paratyphosus* B.

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## STUDIES ON THE CULTIVATION OF THE TYPHUS FEVER RICKETTSIA IN THE PRESENCE OF LIVE TISSUE

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PLATE 24

(Received for publication, December 29, 1931)

This investigation was prompted by the recent significant work of Mooser on the Mexican type of typhus fever. The results on the cultivation of the rickettsia of typhus fever which have already been briefly presented in a preliminary communication (1)\* are reported in detail in this paper.

Various attempts had previously been made by other workers (Kuczynski (2), Krontowski and Hach (3), Wolbach and Schlesinger (4), Rix (5), Zinsser and Batchelder (6) (cf. Zinsser and Castaneda (7))), to cultivate typhus fever rickettsiae. Some of these experiments indicated multiplication of the organisms but none led to the establishment of strains which could be maintained indefinitely *in vitro*. The methods used were practically the same; namely, the cultivation in homologous plasma of tissues (generally brain and spleen) from typhus-infected guinea pigs. In such cultures Kuczynski, Wolbach and Schlesinger, and Zinsser and Batchelder were able to demonstrate rickettsiae morphologically, and Wolbach and Schlesinger succeeded in setting up a second generation, which was done by transferring the same piece of tissue into fresh medium.

Since our preliminary article, Sato (8) reported the cultivation of the virus of typhus fever through thirteen generations, using infected, along with normal Descemet's membrane of rabbits, in a medium consisting of aqueous humor and plasma. The liquid was changed every 2 days and the tissue itself transferred to fresh medium when growth ceased, as it did after from 4 to 10 days. The virulence of such cultures was tested by injecting a suspension of cultivated tissue fragments intracardially. Such injections were followed by fever, monocytosis, and pathological changes consisting of dark red discoloration and edema of the spleen, as well as typhus nodules in the brain, liver, and heart muscle. The author was never able to demonstrate rickettsiae morphologically in his cultures, but described cell inclusion bodies ("*monokokkenförmige Körperchen*") which he identified with the etiological agent in typhus, considering them to be a peculiar form of rickettsia which does not stain with Giemsa or at most very slightly.



Still more recently Pinkerton and Hass (9) described the cultivation of typhus rickettsiae from the testicle of an infected guinea pig. They used as explant material, small fragments of the membranous exudate imbedded in 1 drop of plasma coagulated by 1 drop of embryonic guinea pig tissue extract. The cultures were transplanted every 2 or 4 days by transferring a portion of the tissue into fresh medium. Rickettsiae were demonstrated morphologically in these cultures in histological sections. They state that in the majority of cases, the rickettsiae, whilst numerous in the first generation cultures, disappeared quite rapidly in successive transfers, but in one group of cultures rickettsiae were found in very great numbers in the fourth and fifth generations after 16 and 21 days *in vitro*.

In the following, a description is given of the technic and the media which have been used in carrying cultures of typhus fever rickettsiae for months *in vitro* without diminution either in virulence or in the number of organisms.

### *Technic*

*Typhus Virus*.—The strain of typhus organisms used in these studies (unless otherwise indicated) was isolated from a case in the Southeastern United States by the U. S. Public Health Service in Washington, D. C.<sup>1</sup> It is in all respects quite similar to the Mexican strain of Mooser. A few experiments were also made with a strain from Nicolle's laboratory in Tunis.<sup>2</sup> We have carried the latter strain for some months, transferring sometimes with brain emulsions and sometimes with tunica washings. It may be noted parenthetically, in confirmation of Pinkerton's (10) observations on Wolbach's European strain, that in our hands the Nicolle strain produced, although irregularly, scrotal inflammation of slight to moderate intensity, indistinguishable from that produced by the Mexican type. Rickettsiae, although few in number, could also be demonstrated in the testicular exudate when such was present.

*Media and Cultures*.—Two types of tissue media have been used with equal success. One was adapted from that employed by Rivers, Haagen, and Muckenfuss (11) who used tissue cultures of rabbit cornea in coagulated plasma for the cultivation of the viruses of vaccinia and herpes. For our cultures, pieces (from 2–5 mm. square) of normal tunica from half-grown guinea pigs were soaked in the inoculum suspension, prepared as described below, for 20–30 minutes in order to insure intimate contact between virus and tissue; then, one to three

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<sup>1</sup> We wish to express our gratitude for this material to Dr. G. W. McCoy, Director of the U. S. Public Health Service.

<sup>2</sup> This material was furnished us through the courtesy of Dr. Harry Plotz and Miss Helen Van Sant to whom we are greatly indebted.

pieces were imbedded, according to the directions given by Rivers, Haagen, and Muckenfuss (11), in large tubes (10 cm. long and 2.3 cm. wide) in a small amount of heparinized guinea pig plasma coagulated by means of Ringer solution extracts of normal guinea pig spleen.

The second medium was based on that used by Maitland and Maitland (12), Rivers, Haagen, and Muckenfuss (13), and others for the cultivation of vaccinia virus. This medium consists of Tyrode solution, serum, and minced tissue. For our work, cultures were prepared as follows: Minced normal tunica from half-grown guinea pigs was soaked in a few drops of inoculum for some minutes, after which Tyrode solution and guinea pig serum were added in the ratio of two parts of the former to one of the latter and the mixture distributed in amounts of about 3 cc. into 25 cc. Erlenmeyer flasks.

The tubes and flasks were closed with rubber stoppers and sealed with paraffin to prevent evaporation.

The cultures were incubated at 37.5°C. and transferred at 8-10 day intervals.

*Inoculum for the Cultures.*—To initiate cultures, the tunica containing rickettsiae from an infected guinea pig was scraped in a few cc. of Ringer or Tyrode solution, or ground in a heavy Pyrex 50 cc. centrifuge tube with a glass rod (inserted through a sterile gauze stopper) terminating in a ball deeply cross-hatched to make an effective grinding surface. The slightly turbid fluid thus obtained was used for inoculation. To transfer the cultures from one generation to the next, part of the tissue was removed from the medium and scraped or ground as above with a few drops of the fluid, and the cloudy suspension used to inoculate fresh tissue for several cultures. Tissue fragments were never transferred, only the suspension obtained by scraping.

*Stained Preparations.*—Preparations for staining were made from the cultures by scraping a bit of tissue on a slide with a cataract knife, then spreading the resulting small amount of turbid liquid into a film which was allowed to dry, fixed in methyl alcohol for 2-3 minutes, again dried and stained with alkaline Giemsa in jars. Good staining was obtained in 15-20 minutes, after which time the slides were washed in running tap water, then rinsed with ethyl alcohol and xylene.

Although the Castaneda stain (14) was found to be excellent for demonstrating rickettsiae in the testicular exudate of infected guinea pigs, where the organisms are found largely within the cellular cytoplasm, it did not give as clear pictures with the scrapings of culture material where there were few or no tissue elements serving as background of contrasting color.

*Tests for Virulence.*—To test the virulence of the cultures, tissue was removed from the medium and ground with a small amount of liquid. This, along with the tissue debris, was injected intraperitoneally into guinea pigs. Marked scrotal swelling, characteristic temperature curve, and the subsequent demonstration of rickettsiae in the testicular exudate were used as indications of the virulence of the cultures.

### *Cultivation*

It is apparently quite easy to establish cultures of typhus rickettsiae *in vitro* by means of either of the methods described; namely, the coagulated plasma medium or the serum Tyrode medium. All of six strains initiated by the former method and seven of thirteen by the latter were successful. With more careful selection of infectious material from lesions, the failures could doubtless be considerably reduced.

The tissue fragments imbedded in coagulated plasma began to show outgrowths within 2 or 3 days, reaching the maximum in 5-6 days, the final growth being plainly visible to the naked eye as a halo 1-1.5 mm. wide, surrounding the tissue.

Maitland and Maitland (12) originally believed that their serum Tyrode medium does not contain living cells. Rivers, Haagen, and Muckenfuss (13) have shown that the tissue, although it did not proliferate, was none the less viable for at least 5 days, and capable of proliferation when transplanted into a suitable medium. While it is uncertain whether the tissue in the typhus cultures was still viable at the end of 10 days, the incubation period which was commonly used, the rickettsiae certainly were. No systematic experiments have been made to determine precisely how long the organisms can survive without transfer.

Inasmuch as the coagulated plasma method is somewhat more arduous, it was discontinued in favor of the serum Tyrode medium after the latter was found to support growth as satisfactorily as the former. Cultures have been carried in the latter medium through twenty generations covering a period of 6 months, without diminution in numbers or virulence, and there seems to be little doubt that they can be carried indefinitely as any bacteriological culture.

Chart 1 shows the course of a characteristic infection with the cultivated rickettsiae and indicates the subsequent immunity to the passage strain.

Few to fairly numerous rickettsiae could always be demonstrated in stained preparations from the first generations in both types of cultures, their number increasing in later generations, although there was considerable variation in the number of organisms. Fig. 1 shows the characteristic microscopic picture of the rickettsiae in culture.

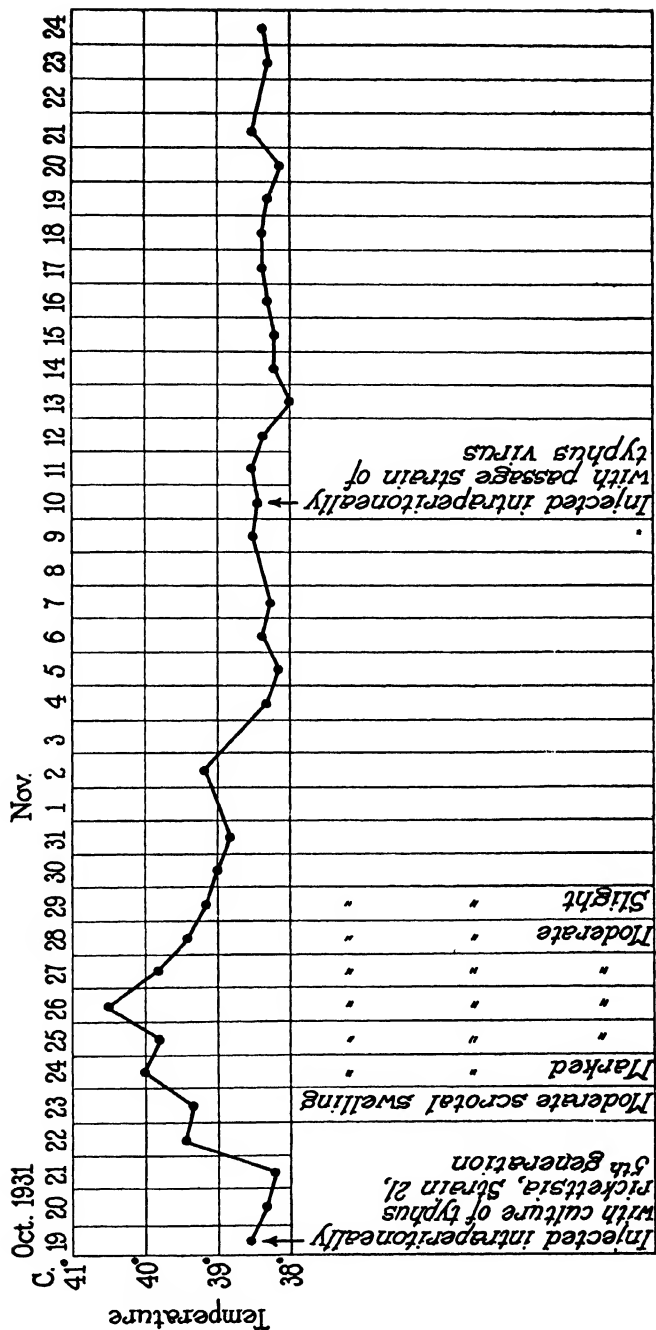


CHART 1. Temperature curve of a characteristic infection with cultivated typhus rickettsiae, showing subsequent immunity to passage strain

The rickettsiae of European typhus were also successfully cultivated from tunica scrapings of an infected guinea pig in the serum Tyrode medium. In the first generations the rickettsiae in these cultures showed a different morphology (see Figs. 2 and 3), *viz.*, a tendency to form chains of varying length, somewhat resembling minute streptococci, resulting in a picture which raises doubt as to the identity of these organisms. However, apart from the fact that there was no growth in the liquid part of the medium, slides from several of the later generations were in all respects similar to those of the Mexican type, and the strain proved to be fully and characteristically virulent on injection into guinea pigs. No growth was obtained on ordinary media with material from this or the Mexican strain.

The fact that the organisms could be demonstrated morphologically in the serum Tyrode medium only in the scrapings from the tissue fragments, and never in the supernatant liquid, would seem to indicate a parasitism of the rickettsiae for the tissue. Guinea pig tests for the infectiousness of the supernatant liquid were equivocal. In this respect the cultures differ from those of some filterable viruses grown in similar media, in that the latter can be transferred by using the liquid (Maitland and Maitland (12), Li and Rivers (15), Rivers (16)).

#### *Experiments with Anaerobiosis and with Heated and Frozen Tissues*

The significance of live tissue is indicated in the results of the following experiments in which heating, freezing, and anaerobiosis were studied as to their influence on the cultures.

The tests for virulence were made with the second generation cultures in the various media, since there was the possibility of a survival of rickettsiae in the inoculum of the first generation.

(a) Minced tunica, suspended in a small amount of Tyrode solution, was heated in a water bath maintained at 50°C. for 15 minutes (*cf.* Pincus and Fischer (17)). This heated tissue was subsequently inoculated and distributed in flasks in the usual manner. Appropriate controls were prepared simultaneously. The tests and controls were transferred after 10 days' incubation, the former again to heated tissue medium, and the latter to unheated tissue medium. Giemsa-stained preparations were made at the time of transfer and again at the end of the second incubation period of 10 days, at which time animals were injected. The results of these experiments are given in Charts 2 *a* and *b*. The numbers in parentheses indicate the generations of the strain.

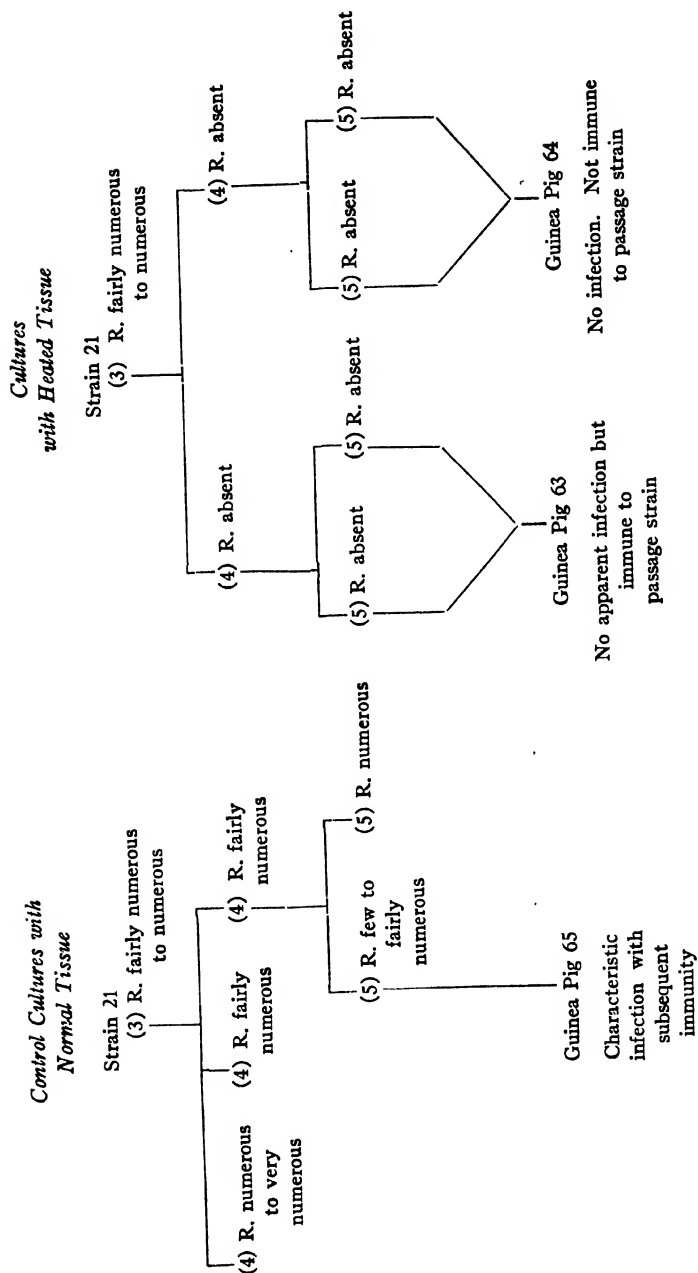
It will be noted that rickettsiae could not be demonstrated microscopically in any of the cultures prepared with heated tissue, either after the first or the second generation. However, of the three guinea pigs inoculated with material from second generations, one had a typical infection and was subsequently shown to be immune to the passage strain. A second guinea pig showed no reaction but was later found to be immune, indicating an unapparent infection, whilst only one of the three showed no signs of infection nor immunity. These results suggest two possible explanations: firstly, that heating at 50° for 15 minutes is slightly less than lethal for the tissue used; secondly, that the rickettsiae can remain viable without apparent multiplication for 20 days after being transferred into the heated tissue medium.

(b) Minced tunica, suspended in a small amount of Tyrode solution, was frozen with CO<sub>2</sub> snow and alcohol and thawed, fifteen times, after which it was inoculated and distributed in the usual manner. Appropriate controls were prepared simultaneously. Transfers, stained preparations, and guinea pig injections were carried out as in the previous experiments with heated tissue. The microscopic findings and the results of animal tests are shown in Chart 3.

It is seen that tissue killed by repeated freezing and thawing failed to support the growth of the rickettsiae.

(c) Normal tunica inoculated in the usual manner was divided between two series of flasks. One series was stoppered and paraffined in the usual manner to serve as controls. The flasks of the other series were identical except for a longer neck into which a two-hole rubber stopper was fitted, carrying one short and one long glass tube, the latter reaching almost to the surface of the medium. Air was replaced in these flasks by passing hydrogen gas through the long tube, the short tube serving as exit. After the air was driven out (5-10 minutes), the long tube was raised (with the stopper still in place) beyond a constriction previously made in the neck of the flask. With the hydrogen passing through, the flasks were sealed at the constriction in an oxygen flame. Transfers were made after 10 days' incubation into media subjected to anaerobiosis as described above, the controls being prepared as usual. The microscopic findings and results of animal tests are shown in Chart 4.

It will be noted that in these experiments the rickettsiae failed to multiply in the tissue medium under strictly anaerobic conditions. If this effect should prove to be constant, it may be attributable either to a deleterious effect on the tissue or directly on the organisms.



R. = rickettsiae.

CHART 2 a

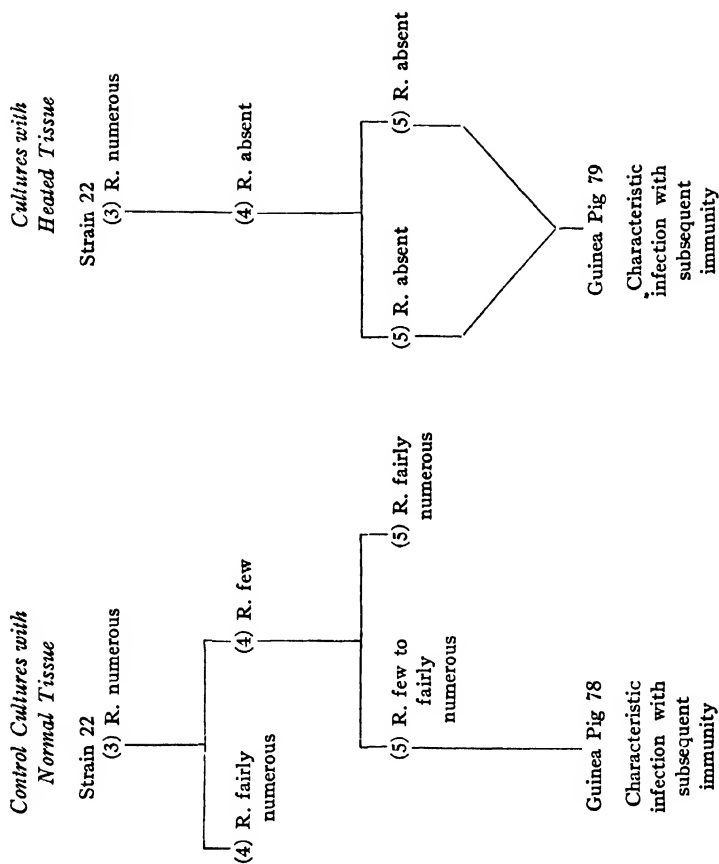


CHART 2 b

CHARTS 2 a and 2 b. Effect of heating the tissue in the culture medium.



*Control Cultures  
with Normal Tissue*

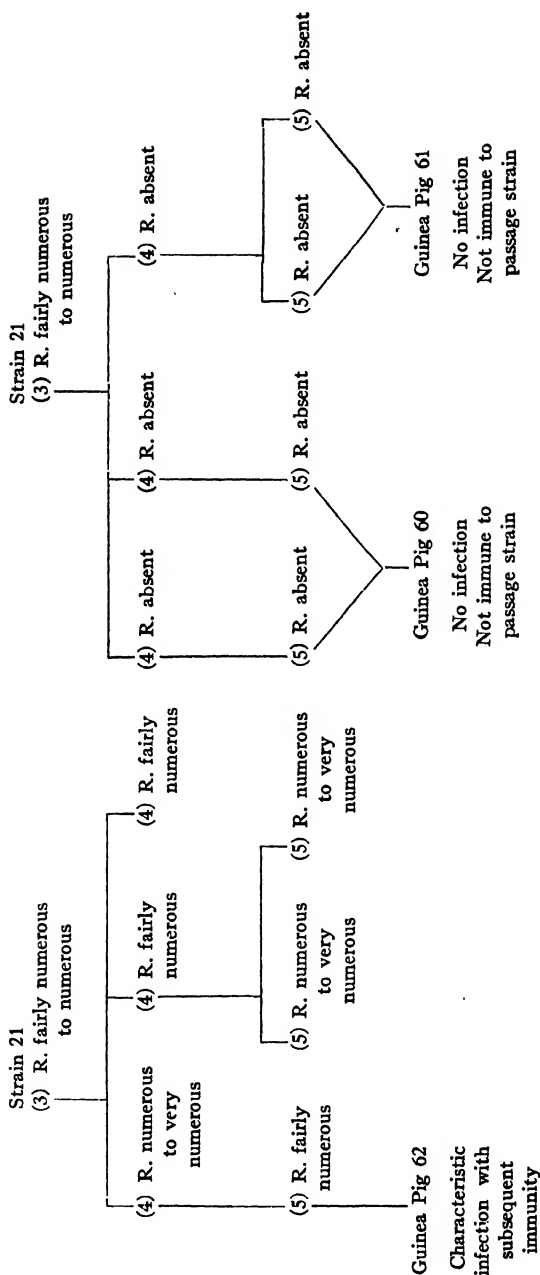


CHART 3. Effect of freezing the tissue in the culture medium. Other experiments with frozen tissue gave identical results.

*Control Cultures Incubated Aerobically*

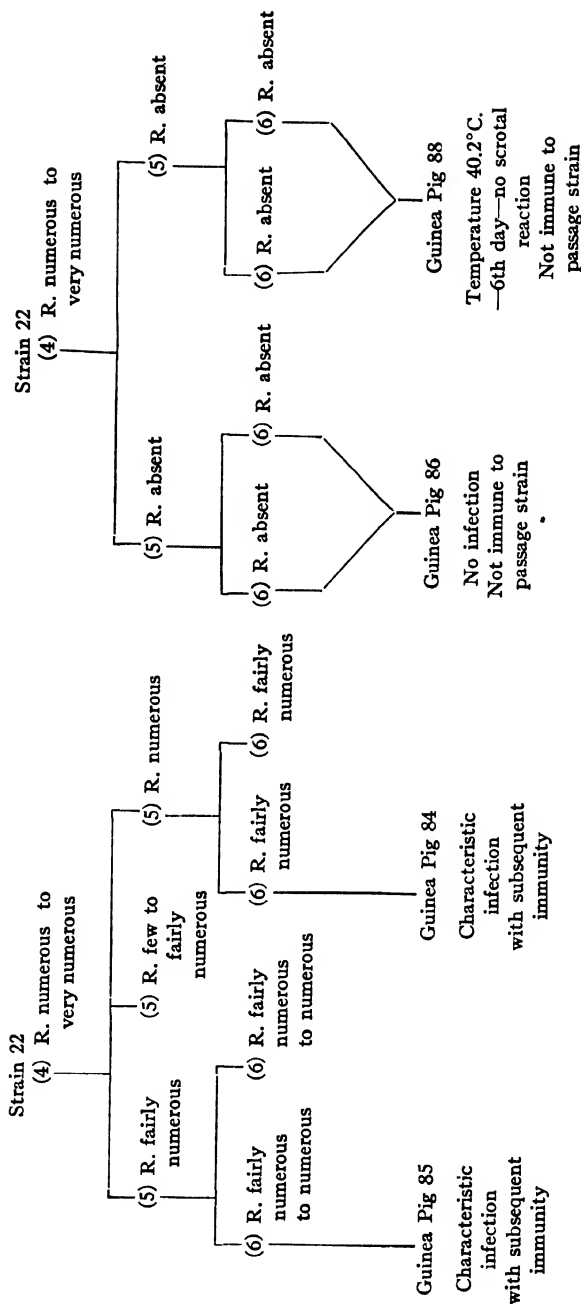


CHART 4. Effect of anaerobiosis on cultures.

Some preliminary experiments were made in order to study further the conditions necessary for cultivation.

It was found that of several tissues tested, only tunica and peritoneum gave satisfactory results in the medium described. The peritoneum, because of its greater surface, offers technical advantages in preparing rickettsia cultures on a larger scale.

A few attempts to cultivate the typhus organisms in a cell-free medium similar to that applied by Eagles and McClean (18) for vaccinia virus, were carried out with centrifuged Tyrode extracts of guinea pig kidney. So far these attempts have been unsuccessful inasmuch as no rickettsiae could be found and no infections could be induced in guinea pigs with these cultures.

Although the preceding results seem to indicate that live tissue is the significant constituent of the media described for the cultivation of the typhus rickettsiae, experiments to maintain cultures in the absence of serum have thus far been unsuccessful. In such experiments a medium was employed consisting only of tissue suspended in Tyrode solution, such as Li and Rivers (15) and Rivers (16) found to be entirely adequate for carrying cultures of vaccine virus. Actually rickettsiae were found in first generations in such a medium but usually reduced in number—and were for the most part absent in the second generations. In one such experiment rickettsiae were demonstrable in two generations, but not in the third. Although serum could not be entirely eliminated from the medium, it was found that the quantity could be reduced to at least half of that used as routine (*i.e.* one part of serum to five parts of Tyrode solution, instead of one to two parts) without damaging the cultures. It seemed to make little difference whether the serum was diluted with Tyrode, Ringer, or ordinary physiological saline. Whether the function of the serum consists merely in prolonging the viability of the tissue, has not been determined.

#### COMMENT

Although the etiological rôle of *Rickettsia prowazeki* in typhus fever hardly needs further confirmation, it is substantiated by the fact that guinea pigs recovered from infections, entirely typical of experimental typhus, produced by the injection of cultures as herein described, are

immune to passage virus. Moreover, rabbits infected with such cultures developed positive Weil-Felix sera.

A significant outcome of the experiments is to be found in the similarity of the growth conditions of *Rickettsia prowazeki* and filterable viruses. In general, the presence of living tissue is considered to be necessary for the cultivation of viruses. This is stressed by Rivers (19) (*cf.* Dale (20)), and was recently substantiated in a paper by Hallauer (21) on the cultivation of the virus of fowl plague (*cf.* Landsteiner and Berliner (22)). This relation of viruses to live tissues has been used as one of the arguments in favor of the view that viruses are not living organisms. It is of interest, therefore, that similar conditions for growth obtain in the cultivation of rickettsiae which, on account of their morphology, certainly must be deemed living microbes.

#### SUMMARY

1. *Rickettsia prowazeki* can be cultivated for many generations *in vitro*, without diminution in numbers or virulence, in media similar to those described by Maitland, Rivers, and others for the cultivation of certain viruses. In all probability, such cultures can be maintained indefinitely.

2. It has been impossible, thus far, to cultivate the typhus rickettsia without employing living tissue.

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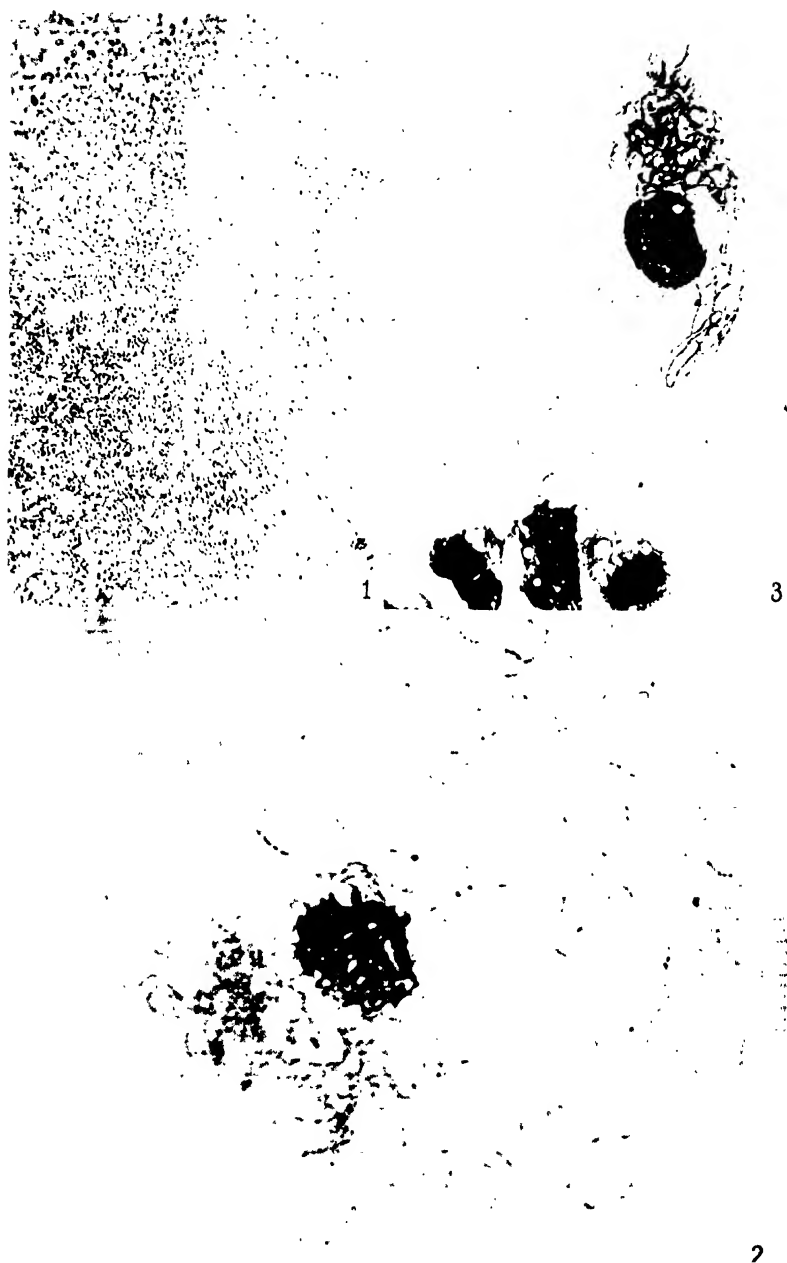
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#### EXPLANATION OF PLATE 24

FIG. 1. Typhus rickettsiae in culture, sixth generation. Giemsa stain.  $\times 1000$ .

FIG. 2. From a culture of European typhus, fourth generation, showing chains intra- and extracellularly. Giemsa stain.  $\times 1500$ .

FIG. 3. From a culture of European typhus, sixth generation, showing long chains intracellularly. Giemsa stain.  $\times 1000$ .





## ON THE SEROLOGICAL SPECIFICITY OF PEPTIDES

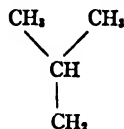
By K. LANDSTEINER, M.D., AND J. VAN DER SCHEER

(From the Laboratories of The Rockefeller Institute for Medical Research)

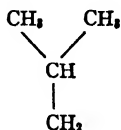
(Received for publication, January 22, 1932)

With the hope of obtaining some information on the precipitin reactions of proteins, experiments have been carried out on the serological properties of antigens whose specificity is determined by the presence of known peptides in the molecule. The preparation of such compounds was attained by the method of combining the substances to be investigated with antigenic protein. Immune sera for these complex antigens were obtained in the usual way. As a first step in the direction indicated, four dipeptides were selected, namely glycyl-glycine, and the inactive forms of glycyl-leucine, leucyl-glycine and leucyl-leucine. From these substances, para-nitrobenzoyl derivatives were prepared by treating them with para-nitrobenzoyl-chloride. The nitrobenzoyl-peptides were reduced, the resulting substances being *p*-aminobenzoyl-glycyl-glycine, -glycyl-leucine, -leucyl-glycine and -leucyl-leucine, as represented by the following formulae:

*p*-Aminobenzoyl-glycyl-glycine:  $\text{NH}_2-\text{C}_6\text{H}_4-\text{CO}-\text{NH}-\text{CH}_2-\text{CO}-\text{NH}-\text{CH}_2-\text{COOH}$

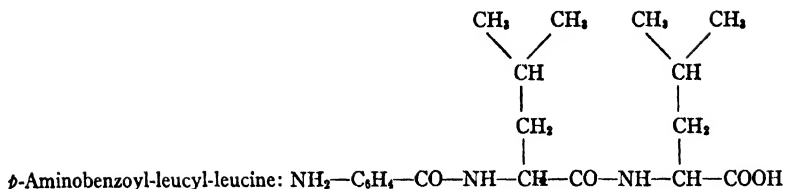


*p*-Aminobenzoyl-glycyl-leucine:  $\text{NH}_2-\text{C}_6\text{H}_4-\text{CO}-\text{NH}-\text{CH}_2-\text{CO}-\text{NH}-\text{CH}-\text{COOH}$



*p*-Aminobenzoyl-leucyl-glycine:  $\text{NH}_2-\text{C}_6\text{H}_4-\text{CO}-\text{NH}-\text{CH}-\text{CO}-\text{NH}-\text{CH}_2-\text{COOH}$





The amino compounds were diazotized and coupled to protein.

For the sake of brevity the aminobenzoyl-peptides will be designated as G.G., G.L., L.G. and L.L. and corresponding abbreviations will be used for the antigens and immune sera.

#### EXPERIMENTAL

*Para-Nitrobenzoyl-Glycyl-Glycine*.—10 gm. of glycine-anhydride were dissolved in 100 cc. of  $N/1$  sodium hydroxide by shaking in a bottle at room temperature. After 20 minutes, when the conversion to glycyl-glycine (1) was completed, the solution was cooled to  $10^\circ\text{C}$ . and during the period of  $\frac{1}{2}$  hour a total of 18.8 gm. of finely ground para-nitrobenzoyl-chloride and 130 cc. of  $N/1$  sodium hydroxide were added alternately in five equal portions, shaking well and keeping the temperature around  $10^\circ\text{C}$ . The filtered alkaline solution, upon addition of an excess of hydrochloric acid gave a crystalline precipitate which was filtered after being kept at  $0-5^\circ$  for 1 hour. The precipitate was washed with cold water, dried and extracted three times with ether to remove any nitrobenzoic acid present (yield 19 gm.). The substance was recrystallized from 40 parts of boiling water, from which it crystallized on cooling as long, narrow, microscopic platelets of light yellow color. Melting point:  $217-218^\circ\text{C}$ . (with darkening), after two more recrystallizations from water. Titration: 0.298 gm. of substance dried at  $100^\circ$  *in vacuo*, dissolved in 80 per cent alcohol, required for neutralization (phenolphthalein) 10.6 cc.  $N/10$  NaOH. Formula  $\text{C}_{11}\text{H}_{11}\text{O}_6\text{N}_3$  requires 10.6 cc.

*Para-Aminobenzoyl-Glycyl-Glycine*.—10 gm. of para-nitrobenzoyl-glycyl-glycine were dissolved in a small volume of water by addition of a slight excess of ammonia, and this solution was added to a hot solution of 65 gm. of ferrous sulfate (7 aq.) in 150 cc. of water. 54 cc. of 28 per cent ammonia solution were added in five portions over a period of 10 minutes, shaking the flask well on each addition. After  $\frac{1}{2}$  hour heating on the steam bath, the ferric hydroxide was filtered off, and the solution was evaporated *in vacuo* to a volume of 75 cc. To this, 500 cc. of alcohol were added, and the salt which separated out was removed by filtration. The alcohol was removed by evaporation *in vacuo* at  $40^\circ$ . To the residue, dissolved in a small volume of water, just enough hydrochloric acid was added to give a maximum precipitation of the amino acid (yield 6 gm.). The substance was recrystallized from hot water from which it precipitated on cooling in long, narrow, microscopic

platelets. The substance turned dark above 220°C. and decomposed without melting.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid:  $C_{11}H_{13}O_4N_3$  calculated N 16.73 per cent, found N 16.49 per cent.

*Para-Nitrobenzoyl-Glycyl-d, l-Leucine*.—10 gm. of glycyl-*d, l*-leucine (2) were dissolved in 300 cc. of water containing 30 gm. of sodium bicarbonate. A total of 15 gm. of finely ground para-nitrobenzoylchloride was added in five equal portions over a period of 1½ hours with vigorous shaking at room temperature. The solution was filtered and acidified with an excess of hydrochloric acid. The sticky precipitate soon crystallized and the crystals were filtered off and washed with water. After drying, the substance was extracted several times with ether (yield 15 gm.), and recrystallized by dissolving in five parts of hot alcohol and diluting the solution with four times its volume of hot water. Upon cooling, the substance crystallized in long, narrow, microscopic platelets of pale yellow color. The melting point was 179–180°C. and did not change after two more recrystallizations from dilute alcohol. Titration: 0.337 gm. of substance dried at 100° *in vacuo*, dissolved in 80 per cent alcohol, required for neutralization 10.05 cc. N/10 NaOH. Formula  $C_{16}H_{19}O_6N_3$  requires 9.99 cc.

*Para-Aminobenzoyl-Glycyl-d, l-Leucine*.—10 gm. of para-nitrobenzoyl-glycyl-*d, l*-leucine were reduced with 54 gm. of ferrous sulfate (7 aq.) and 46 cc. of ammonia solution by the method described for para-aminobenzoyl-glycyl-glycine. As in the latter case, after removal of ammonium sulfate and alcohol, the aminobenzoyl-glycyl-*d, l*-leucine was obtained from a concentrated aqueous solution of the ammonium salt by addition of the appropriate amount of hydrochloric acid (yield 8.5 gm.). The substance was recrystallized by dissolving in 20 parts of boiling water, removing a trace of oily material by filtration and allowing the filtrate to cool slowly. It separated out in long, white, microscopic platelets. Melting point: 83–86°C.

Kjeldahl determination after drying at 60° *in vacuo* over sulfuric acid:  $C_{16}H_{21}O_4N_3$  calculated N 13.68 per cent, found N 13.37 per cent.

*Para-Nitrobenzoyl-d, l-Leucyl-Glycine*.—10 gm. of *d, l*-leucyl-glycine (3) were treated with para-nitrobenzoylchloride as described for the preparation of para-nitrobenzoyl-glycyl-*d, l*-leucine. After filtration, the solution was acidified with hydrochloric acid. A sticky precipitate formed which soon crystallized. The substance was filtered off, washed with water, dried and extracted several times with ether (yield 13 gm.). It was recrystallized by dissolving in six parts of hot alcohol, and diluting the filtered alcoholic solution with 2½ volumes of boiling water. Upon cooling the substance crystallized in pale yellow, thin microscopic needles. The melting point was 205–206°C. and did not change after two more recrystallizations from dilute alcohol. Titration: 0.337 gm. of substance, dried at 100°C. *in vacuo*, dissolved in 80 per cent alcohol, required for neutralization 9.90 cc. of N/10 NaOH. Formula  $C_{16}H_{19}O_6N_3$  requires 9.99 cc.

*Para-Aminobenzoyl-d, l-Leucyl-Glycine*.—10 gm. of para-nitrobenzoyl-*d, l*-leucyl-glycine were reduced with 54 gm. of ferrous sulfate and 46 cc. of ammonia

solution as described before. After removal of the ammonium sulfate with alcohol, the alcoholic solution was concentrated *in vacuo* to a volume of 15 cc. To this solution 25 cc. of methyl alcohol were added and then 200 cc. of ether. Upon standing in the ice box, large, needle-like crystals of pale yellow color formed which consisted mainly of the ammonium salt of the amino acid. No free amino acid separated out from an aqueous solution of this substance when hydrochloric acid was added, and a few attempts to obtain the free amino acids were unsuccessful. The substance lost ammonia on drying *in vacuo* at 60° over sulfuric acid. A sample thus dried to constant weight was found still to contain 1.26 per cent of nitrogen in the form of ammonia.

Kjeldahl determination:  $C_{15}H_{21}O_4N_3$  calculated N 13.68 per cent, found (after subtraction of  $NH_3$  nitrogen) N 13.51 per cent. Melting point: 123–125°C.

*Para-Nitrobenzoyl-d, l-Leucyl-d, l-Leucine A*.—10 gm. of *d, l-leucyl-d, l-leucine A*<sup>1</sup> consisting of a racemic mixture of *d-leucyl-l-leucine* and *l-leucyl-d-leucine* were dissolved at room temperature in 300 cc. of water containing 30 gm. of sodium bicarbonate, by vigorous shaking. After cooling to 0–5°, 100 cc. of chloroform were added and a total of 23 gm. of finely ground para-nitrobenzoylchloride was added in five portions at 20 minute intervals. Shaking was continued, keeping the temperature around 5°C. for  $\frac{1}{2}$  hour after the last addition of nitrobenzoylchloride when no more carbon dioxide was liberated. After addition of 300 cc. of ether and acidification with hydrochloric acid, the insoluble material was filtered and dried. The dry material was extracted several times with ether. It was dissolved in 150 cc. of boiling alcohol, and 150 cc. of hot water were added. After cooling to room temperature crystallization took place. The greater part of the material was dissolved by the addition of enough N/1 sodium hydroxide to make the solution weakly alkaline to litmus. A small part of undissolved substance was removed by filtration, and the filtrate was made acid to Congo by addition of hydrochloric acid. The precipitate was filtered off and dried (yield 9 to 10 gm.). The substance was recrystallized by dissolving in 150 cc. hot alcohol and diluting this solution with an equal volume of hot water. Upon cooling, microscopic needles and square crystals separated out. An investigation showed that the appearance of the two forms of crystals depends upon the conditions under which the crystallization takes place. A recrystallization from 60 per cent alcohol produced almost exclusively square crystals, whereas upon slow crystallization from a more dilute solution in 50 per cent alcohol long fine needles were formed, almost exclusively, which, however, upon longer standing in the mother liquid at room temperature changed completely into the other form. Melting point: 209–210°C. after two further recrystallizations from 50 per cent alcohol. Titration: 0.1965 gm., dried at 100°C. *in vacuo*, dissolved in 80 per cent alcohol, required for neutralization, 4.95 cc. N/10 NaOH. Formula  $C_{19}H_{27}O_6N_3$  requires 5 cc.

*Para-Aminobenzoyl-d, l-Leucyl-d, l-Leucine A*.—10 gm. of para-nitrobenzoyl-*d, l-leucyl-d, l-leucine A* were dissolved in a small volume of hot water with the aid

<sup>1</sup> Prepared by Dr. Marker according to the method of E. Fischer (4. 5).

of a slight excess of ammonia and reduced with 46.5 gm. of ferrous sulfate and 38.7 cc. of ammonia solution as described before. After removal of the ammonium sulfate the alcohol was distilled off *in vacuo* and the volume brought to 100 cc. with water. 21 cc. of N/1 hydrochloric acid were added and the crystalline precipitate was filtered off. This contained some acid-insoluble material and was purified by dissolving in 200 cc. of water by addition of a slight excess of alkali, reprecipitation with hydrochloric acid, addition of an excess of acid, filtration and reprecipitation of the amino acid by the required amount of alkali (yield 4 gm.). The substance was recrystallized by dissolving in 320 cc. of boiling 25 per cent alcohol, from which it separated on cooling as an oil which, upon vigorous stirring, crystallized in white rosettes of microscopic platelets. The substance shriveled at 122°C. and melted at 122–125°C.

Kjeldahl determination after drying at 80° *in vacuo* over sulfuric acid:  $C_{19}H_{29}O_4N_3$  calculated N 11.57 per cent, found N 11.09 per cent.

*Para-Aminobenzoyl-Glycine*.—10 gm. of para-nitrobenzoyl-glycine (melting point: 129–130°C.) (6) were reduced as described before with 72 gm. of ferrous sulfate and 60 cc. of ammonia solution. After removal of ammonium sulfate and alcohol, the concentrated aqueous solution was made weakly acid to Congo by addition of hydrochloric acid. After cooling to 0–5°C. the precipitate was filtered off and dried (yield 7 gm.). It contained a very small amount of acid-insoluble material and was purified by dissolving in 50 cc. of water and 25 cc. of N/1 hydrochloric acid and addition of 25 cc. of N/1 NaOH to the filtered liquid. After standing in the ice box overnight, the precipitate was filtered off and recrystallized from a small quantity of hot water. It separated out on cooling in long, white, microscopic platelets. Melting point: 198–199°C.

Kjeldahl determination after drying at 100°C. *in vacuo* over sulfuric acid:  $C_9H_{10}O_3N_2$  calculated N 14.43 per cent, found N 14.04 per cent.

*Para-Nitrobenzoyl-d,l-Leucine*.—10 gm. of *d,l*-leucine were dissolved in 200 cc. water and 76.5 cc. of N/1 sodium hydroxide; 38 gm. of sodium bicarbonate were added. The solution was stirred vigorously and 21 gm. of finely ground para-nitrobenzoylchloride were added in small portions over a period of 1½ hours at room temperature. The purple colored solution was filtered and made acid to Congo with hydrochloric acid. The precipitate was dried, ground and extracted three times with 200 cc. of ether (yield 14 gm.). It was recrystallized by dissolving in five parts of hot alcohol and diluting the alcoholic solution with an equal volume of hot water. After cooling to room temperature hexagonal crystals separated. Melting point: 229–230°C., after two more recrystallizations from 50 per cent alcohol. Titration: 0.280 gm. of substance dried at 100°C. *in vacuo*, dissolved in 80 per cent alcohol, required for neutralization 10.1 cc. N/10 NaOH. Formula  $C_{13}H_{16}O_5N_2$  requires 9.99 cc.

*Para-Aminobenzoyl-d,l-Leucine*.—10 gm. of para-nitrobenzoyl-*d,l*-leucine were reduced with 65 gm. of ferrous sulfate and 54 cc. of ammonia solution by the usual method. The product obtained was freed from some acid-insoluble material as described for aminobenzoyl-glycine. It was recrystallized from hot water. The

substance formed white microscopic rosettes of needles. Melting point: 182–183°C.

Kjeldahl determination after drying at 100° *in vacuo*:  $C_{13}H_{18}O_3N_2$  calculated N 11.2 per cent, found N 11.25 per cent.

*Chloroacetyl-Glycine*.—The substance was prepared by a method similar to that used by Abderhalden and Hirsch for iodoacetyl-glycine (7). 15 gm. of glycine were dissolved in 200 cc. of *N*/1 NaOH. The solution was cooled in a freezing mixture and a total of 22.6 gm. of chloroacetylchloride and 200 cc. of *N*/1 NaOH were added alternately in five portions, shaking vigorously. The solution was acidified with 40 cc. 5 *N* HCl and evaporated to dryness *in vacuo* at 30°C., and the residue extracted with about 200 cc. of ethyl acetate. The ethyl acetate solution was evaporated on the water bath and kept in a vacuum desiccator. A semisolid crystalline mass formed from which the crystals were separated by filtration on a Buchner funnel. The substance was redissolved in a small quantity of hot ethyl acetate and freed from some insoluble material by centrifugalization. Crystallization took place on standing in the ice box overnight. The crystals (platelets) were filtered off and dried *in vacuo* (yield 12 gm.). Melting point: 92–93°C., after one more recrystallization from ethyl acetate.

Analysis: 0.1162 gm. of substance dried *in vacuo* at 80°C. gave 0.1093 gm. AgCl.  $C_4H_6O_3NCl$  calculated Cl 23.40 per cent, found Cl 23.27 per cent.

*d, l-Leucyl-d, l-α-Amino-n-Butyric Acid*.—10.3 gm. of *d, l-α-amino-n-butyric acid* were dissolved in 50 cc. 2 *N* NaOH. The solution was cooled to 0°. A total of 25.5 gm. of *d, l-α-bromoisocaproylchloride* and 82.5 cc. of 2 *N* NaOH were added in five equal portions alternately over a period of 1 hour, keeping the temperature at about 5°C. and shaking vigorously. 33.7 cc. of 5 *N* HCl were added. The sticky precipitate soon crystallized. It was dissolved in 200 cc. of ether and 4 volumes of petrol ether were added. The precipitate, *d, l-α-bromisocaproyl-d, l-α-amino-n-butyric acid*, was filtered off and dried (yield 16.2 gm.). To convert this substance into *d, l-leucyl-d, l-α-amino-n-butyric acid*, 14 gm. were kept in a well stoppered bottle with 140 cc. 25 per cent ammonia solution for 4 days at room temperature, shaking frequently until the solid material was dissolved. An equal volume of water was added and the solution was evaporated to dryness *in vacuo* at 60°C. Water was again added and the evaporation repeated. The dry residue was extracted three times with 150 cc. hot absolute alcohol to remove ammonium bromide. The remaining 7 gm. of substance were dissolved in 300 cc. of hot 70 per cent alcohol by the addition of ammonia. Ammonia was driven off by boiling and *d, l-leucyl-d, l-α-amino-n-butyric acid* separated out from the hot solution in rhombic microscopic platelets. Melting point: 245–246°C.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid:  $C_{10}H_{20}O_3N_2$  calculated N 12.96 per cent, found N 12.74 per cent.

*Para-Nitrobenzoyl-d, l-Leucyl-d, l-α-Amino-n-Butyric Acid*.—2 gm. of finely ground *d, l-leucyl-d, l-α-amino-n-butyric acid* were suspended in 60 cc. water containing 6 gm. of sodium bicarbonate. The liquid was cooled to 15°C. 20 cc. of chloroform were added and 4.1 gm. of finely ground *para-nitrobenzoylchloride*

in three portions over a period of 1 hour. The mixture was shaken continuously, keeping the temperature below 15°C.  $\frac{1}{2}$  hour after the last addition of nitrobenzoylchloride, 100 cc. of ether were added, and the solution was acidified with hydrochloric acid. The precipitate was filtered off, dried and extracted several times with ether (yield 1.5 gm.). The substance was dissolved in 25 cc. of hot alcohol, some insoluble material was removed by filtration and two volumes of hot water were added to the alcoholic solution. Long, needle-like, microscopic prisms separated on cooling. Melting point: 220–221°C. Titration: 0.181 gm. of substance, dried *in vacuo* at 100°, dissolved in 80 per cent alcohol, required for neutralization 4.90 cc. N/10 NaOH. Formula  $C_{17}H_{23}O_6N_3$  requires 4.96 cc.

*d, l- $\alpha$ -Amino-n-Butyryl-d, l-Leucine*.—13.1 gm. of *d, l*-leucine were dissolved in 50 cc. of 2 N NaOH and the solution was cooled to 0°. A total of 20.4 gm. of *d, l- $\alpha$ -bromo-n-butyrylchloride* and 76 cc. of 2 N NaOH were added alternately in five equal portions over a period of 1 hour, and the liquid was shaken well, keeping the temperature at about 5°C. The sticky precipitate, formed upon addition of 30.7 cc. 5 N HCl, soon crystallized. It was filtered off, dissolved in 15 cc. of ether and four times the volume of petrol ether was added. The substance (*d, l- $\alpha$ -bromo-n-butyryl-d, l-leucine*) separated out in the form of silky needles (yield 16 gm.).

To convert this substance into the amino compound, 14 gm. were allowed to stand in a well stoppered bottle with 140 cc. of 25 per cent ammonia solution for 4 days at room temperature. The isolation of the substance was made as in the case of the *d, l-leucyl-d, l- $\alpha$ -amino-n-butyric acid*. 6 gm. of the substance were obtained. It was recrystallized by dissolving in 100 cc. of alcohol and addition of a quantity of water sufficient to bring about slight precipitation. The solution was then allowed to cool overnight in the ice box. The substance separated in whetstone-shaped crystals (yield 4 gm.). Melting point: 241–242°C.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid:  $C_{10}H_{20}O_3N_2$  calculated N 12.96 per cent, found N 12.60 per cent.

*Para-Nitrobenzoyl-d, l- $\alpha$ -Amino-n-Butyryl-d, l-Leucine*.—2 gm. of *d, l- $\alpha$ -amino-n-butyryl-d, l-leucine* were dissolved in 60 cc. of water containing 6 gm. of sodium bicarbonate. 4.1 gm. of finely ground para-nitrobenzoylchloride were added in three portions at intervals of 30 minutes, the mixture being shaken vigorously. The solution, after filtration, was made acid to Congo by the aid of hydrochloric acid, and the precipitate was filtered, dried and extracted several times with ether (yield 2.5 gm.). The substance was dissolved in 25 cc. of hot alcohol, and an equal volume of hot water was added. Long, thin, needle-like prisms separated on cooling (yield 2.3 gm.). Melting point: 182–184°C. Titration: 0.1856 gm. of substance, dried *in vacuo* at 100°, dissolved in 80 per cent alcohol, required for neutralization 5.15 cc. N/10 NaOH. Formula  $C_{17}H_{23}O_6N_3$  requires 5.08 cc.

*Preparation of the Antigens for Immunization*.—As a representative example, the method used for coupling para-aminobenzoyl-glycyl-glycine to protein will be described. The other substances were coupled to protein in the same way

taking quantities proportional to their molecular weights. 4.8 gm. of para-aminobenzoyl-glycyl-glycine were dissolved in 300 cc. of water and 50 cc. of  $N/1$  HCl, and diazotized with the required amount of sodium nitrite at a temperature of 0–5°C. using starch iodide paper as indicator. The diazo solution was brought to a volume of 720 cc. with ice water. 480 cc. of this solution were added to a cold mixture of 300 cc. of horse serum and 60 cc. normal sodium carbonate and the mixture, cooled with ice, was kept weakly alkaline to phenolphthalein by frequent addition of small quantities of sodium carbonate solution. The coupling proceeded fairly rapidly (tested with alkaline R salt solution for the presence of free diazo compound) and no more free diazo compound was present after 20 minutes. The remaining 240 cc. of diazo solution and 30 cc. of normal sodium carbonate were added (further addition of sodium carbonate as above). The coupling was complete after 20 minutes. The azoprotein was precipitated from the solution by weakly acidifying with hydrochloric acid and after filtration was redissolved in 500 cc. of water by the addition of a small quantity of sodium carbonate solution. It was precipitated from this solution with 6 volumes of alcohol, adding, if necessary, enough dilute hydrochloric acid to obtain coarse flocculation. The precipitated azoprotein was washed with saline, ground in a mortar to a thin paste and brought to a volume of 570 cc. with saline. As a preservative, 30 cc. of a 5 per cent phenol solution were added.

*Immunization.*—Four batches of five rabbits each were taken for the immunizations with the four antigens. Each rabbit received at weekly intervals an intraperitoneal injection of 10 to 15 cc. of the antigen. Two active sera were obtained in each batch of animals.

*Antigens for the Test.*—The azoproteins for the test solutions were prepared in the same way as those used for the immunization but with twice the amount of diazotized substance; e.g., in the case of para-aminobenzoyl-glycyl-glycine, 0.32 gm. was diazotized and coupled with 10 cc. of serum. (The treatment with alcohol was omitted.) In this manner test antigens were prepared from chicken serum, ox serum and horse serum. The dilutions given in the tables are in terms of a 5 per cent stock solution. The intensity of the reactions is indicated as follows: 0, f.tr. (faint trace), tr. (trace),  $\pm$ ,  $\pm$ ,  $+$ ,  $+\pm$ , etc.

### *Precipitin Tests*

The specificity of the immune sera was tested with the antigens prepared from chicken serum. The results obtained are shown in Table I. The other immune sera not recorded in the table gave practically identical results.

From the table it appears that the reactions of the G.G., G.L. and L.G. immune sera were specific for the homologous antigens. Also the L.L. immune serum gave the strongest reaction with the corre-

TABLE I  
To 0.2 cc. of the diluted antigens (prepared with chicken serum) were added 2 capillary drops of immune serum.

[illegible]



sponding antigen, but in addition a distinct precipitation with the G.L. antigen also containing leucine as the terminal amino acid. When the tests were set up with larger quantities of immune serum, similar cross-reactions occurred with the G.L. and L.G. immune sera (Table II); the greater influence of the terminal group on the specificity is also shown in tests with antigens made with ox serum (Table III). This effect was noticeable but not as marked in tests with an-

TABLE II

To 0.2 cc. of the 1:500 diluted antigens (prepared with chicken serum) were added 5 drops of immune serum.

Immune sera	Readings taken after	Antigens			
		G. G.	G. L.	L. G.	L. L.
G. G.	1 hr. at room temperature	++	0	0	0
	2 hrs. at room temperature	++±	0	0	0
	Night in ice box	++++	0	tr.	0
G. L.	1 hr. at room temperature	0	++	0	f.tr.
	2 hrs. at room temperature	0	++±	0	tr.
	Night in ice box	0	++++	0	+
L. G.	1 hr. at room temperature	f.tr.	0	++	0
	2 hrs. at room temperature	+	0	+++	0
	Night in ice box	+	0	++++	0
L. L.	1 hr. at room temperature	0	±	0	+
	2 hrs. at room temperature	0	+	0	++
	Night in ice box	0	++	0	+++

tigens prepared from horse serum, obviously because of the prevailing influence on the reactions of the protein part of the antigen.

It is striking that the ox antigens reacted more intensely with the "heterologous" sera than the chicken antigen. Evidently, the heterologous reactions of the ox antigen are caused by a decrease in species specificity following the combination with azo compounds, which brings out the relationship between ox protein and the horse protein used for immunization, whereas there were no reactions with the avian azoproteins which do not contain the same terminal amino acid as the immunizing antigen.

TABLE III  
To 0.2 cc. of the diluted antigens (prepared with ox serum) was added 1 drop of immune serum.

Immune sera	Antigens .....	G. G.				G. L.				L. G.				L. L.			
		Dilution				Dilution				Dilution				Dilution			
		1:100	1:500	1:2,500	1:12,500	1:100	1:500	1:2,500	1:12,500	1:100	1:500	1:2,500	1:12,500	1:100	1:500	1:2,500	1:12,500
G. G.	Readings taken after	±	+	±	0	f.tr.	0	0	0	tr.	tr.	0	0	0	0	0	0
	15 min. at room temperature 1 hr. at room temperature	+	+	+	tr.	tr.	0	0	0	±	±	f.tr.	0	0	0	0	0
G. L.	15 min. at room temperature 1 hr. at room temperature	0	f.tr.	0	0	±	+	±	0	0	0	0	0	tr.	±	tr.	0
		±	±	±	tr.	±	+	+	±	±	±	±	0	tr.	+	±	tr.
L. G.	15 min. at room temperature 1 hr. at room temperature	±	±	tr.	0	0	0	0	0	±	+	±	0	0	0	0	0
		±	±	±	0	tr.	0	0	0	±	+	±	0	0	0	0	0
L. L.	15 min. at room temperature 1 hr. at room temperature	tr.	±	0	0	±	+	±	0	±	±	0	0	±	±	±	0
		±	±	tr.	0	±	+	±	0	±	+	tr.	0	±	±	+	0

After the tests had been allowed to stand overnight in the ice box, the specificity was indistinct.

Apart from the reactions described which show a definite regularity, the following minor observations may be recorded: In high concentrations (1:20) of the chicken antigens, weak non-specific reactions occurred with the L. L. antigen (see (8)).

When small amounts of immune serum were used (1 drop of immune serum + 0.4 cc. 1:100 or 1:500 chicken antigen, weak or faint reactions appeared slowly and with some sera did not show marked specificity.

The immune sera were also tested with azoproteins prepared from para-aminobenzoyl-glycine and para-aminobenzoyl-*d*, *l*-leucine by coupling to chicken serum. Distinct precipitations were obtained by action of the G.G. and L.G. immune sera on the *p*-aminobenzoyl-glycine antigen, but the same antigen gave weak reactions also with the G. L. and L. L. immune sera. These phenomena were not investigated further and do not at present permit of a simple explanation.

### *Inhibition Tests*

The specificity displayed by the inhibition tests with the aminobenzoyl- and nitrobenzoyl-dipeptides parallels that of the precipitin reactions (Tables IV, V, VI). Of the non-acylated peptides tested, namely glycyl-glycine, *d*, *l*-leucyl-glycine and glycyl-*d*, *l*-leucine, only the last substance showed a distinct inhibiting action on the precipitin reactions; *viz.*, those of the G.L. and L.L. immune sera. Chloroacetyl-glycine had a greater inhibitory effect than glycyl-glycine on the reactions of the G.G. immune serum, and similarly chloroacetyl-leucine inhibited the action of the G.L. and L.L. immune sera more than glycyl-leucine. The action of para-aminobenzoyl-glycine and para-aminobenzoyl-*d*, *l*-leucine is similar to that of the chloroacetyl compounds. In general, leucine in the terminal position seems to have more significance than glycine, perhaps on account of the larger size of this amino acid. *d*, *l*- $\alpha$ -Bromoisocaproyl-glycine inhibited the reactions of all four immune sera, most markedly that of the L.G. serum.

### DISCUSSION

The four peptide-azoproteins examined proved to be readily distinguishable by precipitin tests, each serum giving the strongest reaction with the homologous antigen; only in the case of the L.L. immune serum there was little difference between the reactions on the L.L. and G.L. antigens. It can be concluded, therefore, that the nature of both amino acids present in the peptides examined, as well as their position,

determines the specificity, the length of the chain being the same in all four compounds. Consequently, the amino acids may be looked

TABLE IV

In this and in the following tables, 0.2 cc. of the chicken antigen (diluted 1:500) was mixed with 0.05 cc. of a neutral solution of the substances indicated (concentration 1 millimol in 10 cc. in the tests in Tables IV and VI). To this 2 capillary drops of the homologous immune sera (3 drops of the L. L. immune serum) were added. The control tube contained only antigen and immune serum. The tests were allowed to stand at room temperature and afterwards overnight in the ice box.

Immune sera	Readings taken after	Substances used for the inhibition tests*									
		<i>p</i> -Aminobenzoyl-glycyl-glycine	<i>p</i> -Aminobenzoyl-glycyl- <i>d</i> , <i>l</i> -leucine	<i>p</i> -Aminobenzoyl- <i>d</i> , <i>l</i> -leucyl glycine	<i>p</i> -Aminobenzoyl- <i>d</i> , <i>l</i> -leucyl- <i>d</i> , <i>l</i> -leucine	Glycyl-glycine	Glycyl- <i>d</i> , <i>l</i> -leucine	<i>d</i> , <i>l</i> -Leucyl-glycine	Chloroacetyl-glycine	Chloroacetyl- <i>d</i> , <i>l</i> -leucine	Control
G. G.	15 min.	0	0	0	f.tr.	±	±	±	0	tr.	±
	1 hr.	0	±	0	±	+	±±	+	tr.	+	+
	3 hrs.	0	±	f.tr.	+	±±	±±	±±	±	±±	+
	Night in ice box	0	±±	±	±±	+++	++	++	±±	+++	++
G. L.	15 min.	tr.	0	f.tr.	0	±	f.tr.	±	tr.	0	tr.
	1 hr.	±	0	±	0	+	tr.	+	±	0	±
	3 hrs.	+	0	±	0	±±	±	±±	±±	0	±±
	Night in ice box	+	0	+	tr.	±±	+	±±	++	tr.	++
L. G.	15 min.	0	0	0	0	+	+	±	±	±	+
	1 hr.	f.tr.	±	0	f.tr.	±±	±±	+	±	±	+
	3 hrs.	±	±±	0	±	±±±	++	±±	++	++	++
	Night in ice box	±±	++	0	±±	±±±	±±±	++	±±±	±±±	+++
L. L.	15 min.	0	0	0	0	±	0	tr.	0	0	0
	1 hr.	f.tr.	0	0	0	±±	0	+	±	0	±
	3 hrs.	±	0	f.tr.	0	++	tr.	++	+	0	+
	Night in ice box	±±	0	+	0	+++	±±	++	++	tr.	++

\* The *d*, *l*-leucyl-*d*, *l*-leucine preparation could not be used for the test on account of its sparing solubility in neutral solution.

TABLE V

In these tests a smaller amount of the substances was used, namely 0.05 cc. of a neutral solution containing 0.25 millimol in 10 cc.

Readings taken after	Immune serum	Substances			Immune serum	Substances			Immune serum	Substances		
		<i>p</i> -Aminobenzoyl-glycyl-glycine	<i>p</i> -Aminobenzoyl-glycyl- <i>d</i> , <i>l</i> -leucine	Control		<i>p</i> -Aminobenzoyl-glycyl- <i>d</i> , <i>l</i> -leucine	<i>p</i> -Aminobenzoyl- <i>d</i> , <i>l</i> -leucyl- <i>d</i> , <i>l</i> -leucine	Control		<i>p</i> -Aminobenzoyl-glycyl- <i>d</i> , <i>l</i> -leucine	<i>p</i> -Aminobenzoyl- <i>d</i> , <i>l</i> -leucyl- <i>d</i> , <i>l</i> -leucine	Control
1 hr.	G. G.	0	±	±±	G. L.	0	0	±±	L. L.	f.tr.	0	+
2 hrs.		0	+	±±		0	tr.	±±		f.tr.	0	+
3 hrs.		0	+	±±		0	±	±±		f.tr.	0	±±
Night in ice box		0	±±	++		0	±	++		tr.	0	++

TABLE VI

Immune sera	Readings taken after	Substances used for the inhibition tests						
		<i>p</i> -Nitrobenzoyl-glycyl-glycine	<i>p</i> -Nitrobenzoyl-glycyl- <i>d</i> , <i>l</i> -leucine	<i>p</i> -Nitrobenzoyl- <i>d</i> , <i>l</i> -leucyl-glycine	<i>p</i> -Nitrobenzoyl- <i>d</i> , <i>l</i> -leucyl- <i>d</i> , <i>l</i> -leucine	<i>p</i> -Nitrobenzoyl- <i>d</i> , <i>l</i> -leucyl- <i>d</i> , <i>l</i> -α-amino-butyric acid	<i>p</i> -Nitrobenzoyl- <i>d</i> , <i>l</i> -α-amino-butyryl- <i>d</i> , <i>l</i> -leucine	Control
G. G.	1 hr.	0	+	tr.	+	+	+	+
	3 hrs.	0	±±	+	++	±±	+	++
	Night in ice box	0	++	±±	++	++	++	+++
G. L.	1 hr.	±	0	±	0	0	0	+
	3 hrs.	+	0	+	0	tr.	0	±±
	Night in ice box	++	0	±±	tr.	+	tr.	+++
L. G.	1 hr.	f.tr.	+	0	tr.	tr.	±	+
	3 hrs.	±	±±	0	±	±	±	++
	Night in ice box	++	+++	0	++	++	++	+++
L. L.*	1½ hrs.	±±	tr.	+	0	±	0	±±
	3 hrs.	++	±	±±	0	±	0	++
	Night in ice box	+++	±±	++	0	±±	±	+++

\* For the test with the L. L. immune serum, solutions containing only 0.25 millimol instead of 1 millimol of the substances in 10 cc. were added.

upon as units, each having an influence on the serological reactions. Since the cross-reactions were stronger when the terminal groupings were the same than in the other two instances in which the amino acids next to the end were identical, it seems that the amino acids carrying the free carboxyl are the more significant, which agrees with previous results (8) pointing to a predominant influence of acid groups. The fact that both components of the dipeptide have a distinct influence on the reactions recalls observations by Balls and Köhler who found an analogous condition in the splitting of peptides by dipeptidase (9) and carboxypolypeptidase (10). On the basis of their results and in accordance with similar theoretical assumptions of von Euler (11), they suppose a double attachment of these ferments; thus dipeptidase would combine not only with the free  $\text{NH}_2$  but also with the  $\text{NH}$  group of dipeptides.

It remains to be decided whether such an assumption is valid for the reactions under discussion, or whether the explanation is to be found in a modification of the specificity of the binding groups by neighboring structures. Another alternative implying the action of several antibodies requires further investigation. It will obviously be of interest to extend the present studies to longer peptide chains in order to determine if in such compounds as well the replacement of one amino acid by another would be detectable. Mention has already been made of the parallel results observed in the inhibition reactions and the precipitin tests. The stronger inhibition by the nitro- or aminobenzoyl compounds as compared with the non-acylated dipeptides is in accordance with previous observations (12) on immune sera specific for antigens prepared with diazotized para-aminotartranilic acid. The reactions of these immune sera were much more markedly inhibited by nitro- and aminotartranilic acid than by tartaric acid. As an explanation it has been suggested that the antibody combines not only with the tartaric acid radical but, by virtue of another binding group, also with the aromatic part of the inhibitory substances. Although this assumption may be valid and may apply also to the present case, there are factors other than the structural correspondence which influence the reactions. This is seen from the observation that chloroacetyl-glycine and chloroacetyl-*d*,*l*-leucine are more active than glycyl-glycine and glycyl-*d*,*l*-leucine, respectively, in inhibition

tests made with the G.G. and G.L. immune sera. These results show that the inhibitory effect of the amino acid is enhanced by the introduction of an acyl group which is probably due to an increase in the acidity of the binding groups. A similar view was expressed by Waldschmidt-Leitz on the action of carboxypolypeptidase on chloroacetyl-tyrosine in comparison with glycyl-tyrosine (13). The questions raised invite further investigation. Other lines along which a continuation of the present experiments seems desirable, are the examination of peptides built up from a greater choice of amino acids, the use of optically active amino acids and of peptides with free amino groups or with esterified carboxyl groups.

#### SUMMARY

With the idea that studies on the serological properties of peptides may ultimately aid in the understanding of the precipitin reactions of proteins, antigens have been prepared containing aminobenzoylated dipeptides, namely glycyl-glycine, glycyl-*d*, *l*-leucine, *d*, *l*-leucyl-glycine and *d*, *l*-leucyl-*d*, *l*-leucine. These four antigens were found to be different serologically, their specificity depending on the structure of the terminal amino acid carrying the free carboxyl group, and to a less degree also on the second amino acid. The results were obtained by means of precipitin and inhibition tests. Analogies to observations on enzyme specificity are discussed.

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## THE VESSELS INVOLVED IN HYDROSTATIC TRANSUDATION

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### PLATE 22

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The permeability of the walls of the cutaneous venules markedly exceeds that of the capillaries (1). Can it be that under circumstances of pathologically increased venous pressure, as in heart disease or when a limb is constricted, edema develops by a transudation which is localized, primarily at least, to the venules? We have sought to answer this question by testing the influence of slight increases of the venous pressure upon the region and rate of escape of materials from the cutaneous vessels, as indicated by the passage outwards of vital dyes devoid of complicating affinities.

Chicago blue 6B and pontamine sky blue were selected for the tests because the gradient of vascular permeability which importantly conditions the spread from the blood is readily demonstrable with them (2). They pass out of the vessels slowly, but their color is so intense that local differences in the rate of escape are plainly to be discerned. The ear of the mouse was used because the course of events can be followed directly in it; and its veins were obstructed to the desired degree by means of an apparatus developed for the purpose.

### *Method*

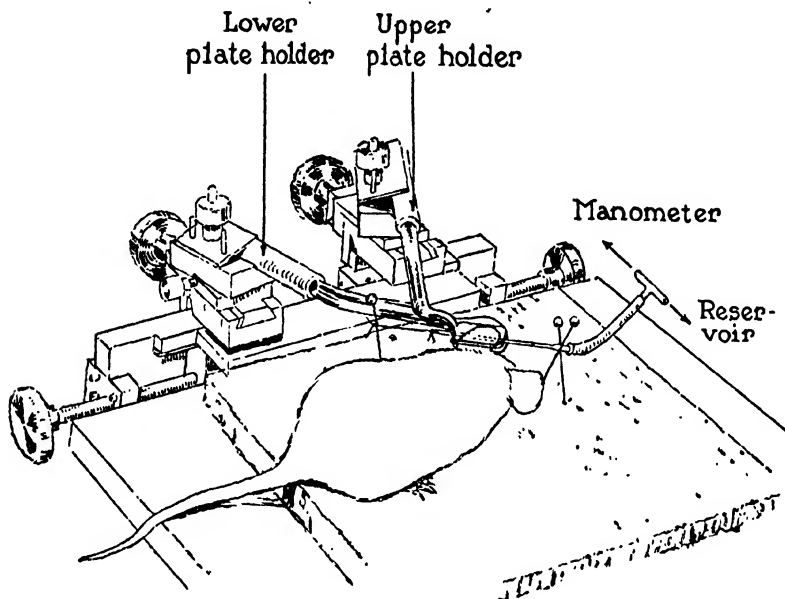
The ear was viewed in paraffin oil between parallel glass plates; and before or after the injection of dye into the blood stream the vessels were obstructed near the middle of the organ by means of pressure exerted through a collodion bag.

A rhomboidal platform of white porcelain (1.5 x 2 cm.), fused to the end of a glass rod, is fixed horizontally in one clamp of a Chambers microdissection apparatus, over a cork animal board. On top of the platform, just inside its narrow free edge, is placed a slender, sausage-shaped, collodion bag, about 3 cm. long and 2 mm. in diameter when full of water. The bag connects by a water filled glass



cannula and rubber tubing with a manometer in which the water has been stained to make the readings easier. A side arm leads to a reservoir and a pump where-with the manometer column can be raised or lowered very rapidly to any desired level. A looped thread is tied to the free end of the collodion bag and the latter is held in place with pins thrust into the animal board through this loop, and with others set to either side of the cannula (Text-fig. 1).

The mouse, under sodium luminal,—0.2 to 0.23 cc. of a 2 per cent solution for a



TEXT-FIG. 1. A diagrammatic sketch of the apparatus used for obstructing veins or arteries in the ear of the mouse.

The rhomboidal platform of white porcelain, fused to the end of a glass rod, is placed in the lower plate holder of a Chambers microdissection apparatus. The slender collodion bag stretches across the lower plate and is connected by glass and rubber tubing with the manometer, reservoir, and pump where-with the manometer column can be raised to any desired level. The ear of the mouse lies on the rhomboidal platform and above the bag. A glass platform similar to the porcelain one is fixed in the upper plate holder of the Chambers apparatus and brought down over the ear.

20 gm. animal, given subcutaneously an hour or two beforehand,—is placed on its belly with the head next the platform, and the latter is so adjusted that the ear rests upon the bag with nearly half projecting beyond. Ear, platform, and bag are now flooded with neutral paraffin oil of low viscosity, and any skin folds are

smoothed out with a camel's hair brush before another platform, smaller than the first and of transparent glass, is brought down upon the preparation with the aid of the Chambers apparatus. The platforms should be parallel and as far apart as is compatible with compression on filling the bag. The oil renders the hairs invisible when the cooled light from an arc is properly directed by a plane mirror with a universal joint. The blood vessels immediately over the bag, as well as those beyond it, stand out so clearly that both the direct and indirect effects of the pressure changes can be followed with the binocular dissecting microscope. The direct effects are localized to a strip of tissue not more than 1 mm. wide. Only 0.3 cm. of water pressure is necessary to distend the bag, and 1 to 2 cm. causes a visible denting of the walls of the large veins of the ear where pressed upon.

The bags are made out of a 4 to 5 per cent collodion solution in ether and alcohol. Small glass rods coated with caramelized sugar serve as forms; and the sugar is dissolved in 95 per cent alcohol after the bags have dried sufficiently for use. They can be stored in alcohol. When properly made they are flexible, and leak only a negligible amount of water at pressures of 70 to 100 cm.

The bore of the manometer employed is only 0.5 mm.; but since all pressure determinations are relative to a base line determined to some extent by capillarity, this latter factor can be ruled from account. The pressure lag in the bag and the elastic rebound of the rubber connecting tubes can be minimized by avoiding jerky pressure changes.

Comparisons with the free ear of the animal have shown that when the obstructing bag is empty and the platforms at the optimum distance apart for pressure determinations the distribution of dyes is identical in the two. If the bag is close to the head, pressure from it may cause angulation of the cartilage and vessels with untrustworthy results. If it overlies the ear, it sinks irregularly into the soft tissue on distention, stretching and distorting the vessels and often closing some without affecting others of like sort. But when it is on the under side of the organ about half-way to the tip, its pressure is exerted evenly on the cartilage through a thin skin layer, and the ear vessels of the upper side, being pressed between the cartilage and the overlying glass plate, are closed off without evident traction upon them or distortion of the structures to either side. The pressure conditions under such circumstances resemble those with the distensible arm band used clinically. By direct inspection of the vessels one can tell within 1 to 2 cm. how great a column of water is required to shut off veins or arteries.

One worker manipulated the pressure apparatus, a second watched the vascular changes, and a third made notes. Only 2 to 3 seconds were required to bring the column in the manometer to the maximum height required for arterial occlusion, 70 cm., and a much shorter time when lower pressures were worked with.

### *The Arterial and Venous Pressures in the Mouse Ear*

In a preceding paper the vascularization of the mouse ear has been described in detail (1). Its main vessels course upon the outer side

Always the stained blood took the way of the capillary web. It seems safe to conclude that effective arteriovenous anastomoses do not exist in the ear of the mouse.

When suddenly blocked the veins do not at once widen. The rapid mounting of pressure behind the obstacle presented by the collodion bag might conceivably be due to a fixation of the capillaries in tissue which does not give. But when the ear is touched on its upper surface with a rounded glass point the tissue is seen under a magnifying glass to dimple and to be loose, while furthermore there is room in it for the rapid accumulation of much edema fluid. This happens when a pressure is exerted upon the veins that is sufficiently high to shut them off while the arteries still pump blood in. Only when the obstacle to outflow is so considerable does capillary dilatation become well marked. A pressure which suffices merely to narrow the veins and to interfere to some extent with flow through them, as shown by a more rapid current where the bag indents the vessel, causes, it is true, some capillary widening as can be seen when the vessels distal and proximal to the bag are compared after the blood has been darkened with dye. But this widening is very slight. Krogh has stressed the fact that some of the contracted capillaries, of muscle especially, are not opened by great pressure (5), and Tannenbergs and Fischer-Wasels state that after veins have been tied off the capillaries do not at once dilate (8). The present work shows that the patent capillaries of the mouse ear do not immediately give way when subjected to arterial pressure but transmit this with but little loss to the venous blood.

#### *Effect of Increased Venous Pressure on the Escape of Substances from the Blood Stream*

The opportunity for vital dyes to escape from the blood into the corium of mouse skin, whether of the ear or of the body, increases along the further portion of the capillary web and is greatest in the region of the primary venules. This is not because of a more favorable ratio of wall surface to vascular content. It results from intrinsic local differences in vascular permeability. During the first minutes after an intravenous injection of Chicago blue 6B bright blue patches develop in the corium of the ear, owing to an escape of dye from the further part of the capillary web and the adjoining venules before

any gets out elsewhere. Eventually the staining becomes uniform owing to a redistribution within the tissues. With the more poorly diffusible pontamine sky blue the patches develop more slowly and are smaller, while with highly diffusible dyes (brom phenol blue, patent blue V) some general coloration develops at the same time as the patching, because the capillary web is everywhere permeable to these dyes, though unequally so. These findings have been illustrated in a preceding paper (1).

In the present experiments a known degree of obstruction to the venous outflow was produced, Chicago blue or pontamine blue was injected, and the ear compared with its fellow. The course of the staining was followed under the microscope in the organ that was pressed upon, differences in the distribution of stain to the tissue proximal and distal to the pressure bag being readily seen. At various times after the injection both ears were lopped off, the pressure was only then relaxed, and the specimens were compared while side by side in oil under a single cover slip with their proximal halves, wherein no difference could be expected, blocked from sight with squares of white paper.

When the systolic pressure was very low, the ear with partially obstructed veins did not stain as well as the control because the arteries were compromised by the pressure exerted, as could be directly observed, and the turnover of stained blood was cut down. All such instances were discarded. In mice with a vigorous circulation (and a venous pressure of 18 to 23 cm.) it was found that a water column 5 to 6 cm. high was required to narrow the veins sufficiently for recognizable interference with outflow. Slighter pressures caused some denting of the veins but the latter were still so broad that there was no visible change in the blood current; and the staining in such instances did not differ from that in the control organ. Pressures of 5 to 6 cm. had pronounced results on the staining. Pontamine blue and Chicago blue began to escape at once from the small venules of the ear and the further portion of the capillary web, with result that the characteristic, patchy staining was already marked at a time when no dye had emerged in the region proximal to the pressure bag and none in the control ear. The subsequent coloration was more intense than in the control tissue. Its distribution in relation to the vessels was not altered from the ordinary, however. With pressures of 12 and 13 cm., which did not prevent an abundant rapid flow through the veins though compressing them to half size or less, the ear again became patched with blue more quickly and intensely than its fellow, and the patches were larger than ordinary, because dye escaped into the tissues further back along the capillary web in the

direction of the arterioles. In addition a narrow zone of deep blue formed along the larger veins, in some cases even along the largest (fan veins), and there was some diffuse staining of the ear as a whole before any developed in the control. In animals having a very low arterial blood pressure, and excluded for such reason, some of these differences in distribution could be noted despite the fact that the staining was less intense than that in the control ear.

The findings show that a slight interference with venous outflow enhances the escape of dye through the walls of the venules and the adjoining portion of the capillary web. When the interference is more considerable these effects extend further back along the capillary web, and veins not ordinarily permeable let dye through. A typical result of such interference has been photographed in Fig. 1. Even when the changes are very marked the color pattern still indicates that the opportunity for escape from the blood is greatest in the region of the primary venules and least in the proximal part of the capillary web, that near the arterioles.

As already mentioned, the smallest pressure increase causing an evident obstruction to venous outflow gave rise also to a perceptible widening of the capillaries. This was unaccompanied by any pronounced increase in their general permeability, a fact sufficiently attested by the unaltered though accentuated staining pattern. With a sustained pressure obstacle of 12 to 13 cm. of water, distention of the capillaries was considerable and edema of the ear developed, as shown by thickening of it, pitting under pressure, and an almost complete emptying of the vessels when the organ was cut off. The ear colored rapidly but the existence of a gradient of distribution was plainly to be perceived.

In an accessory group of experiments the maximal venous obstruction compatible with flow was produced,—that is to say, the pressure in the bag was raised to within a few centimeters of the systolic arterial pressure and maintained for a few minutes. During this period the capillary web became greatly distended and solid columns of red cells formed in some of the meshes. Now dye was injected. It at once passed out everywhere along the capillary web, except from the blocked meshes, and a deep, generalized staining rapidly developed, without trace of the pattern indicative of the ordinary gradient of capillary and venular permeability. Staining of the same sort occurred when

the pressure obstacle had been done away with just prior to introducing the dye, as also when the local circulation was stopped by bag pressure just after the stained blood had been distributed through the vessels. Evidently the distention of the capillaries was accompanied by a great increase in the permeability of their walls, a change which was not immediately reversible.

When an anesthetized mouse is suspended head downwards, the arterial and venous pressures in the ear mount rapidly and the organ becomes engorged with bright blood. We have followed the phenomena with the anesthetized animal hanging by the hind legs over a platform on which the ear is spread as usual. Repeated rapid distension of the collodion bag causes the pressure to rise under ordinary conditions, but it is a far more effective stimulus when the animal hangs head downward. There is a prompt return to ordinary pressures, however, when the mouse is placed once again on its abdomen. These facts can be illustrated by the following protocol.

Mouse weighing 18 gm., given 0.21 cc. of a 2 per cent solution of sodium luminal into the subcutaneous tissue  $1\frac{1}{4}$  hours prior to the experiment.

The venous pressure in the ear with the animal on its abdomen was 21 cm.  $H_2O$  and the systolic arterial pressure 3 minutes later 40 cm. Now the mouse was hung head downwards for  $12\frac{1}{4}$  minutes. The venous pressure at the end of this time proved to be 27 cm.  $H_2O$ , with an arterial pressure of 65 cm. and jerky flow at 62 cm. A pressure of 30 cm. exerted  $1\frac{1}{4}$  minutes after this last reading failed to shut off the veins and so too did 35 cm. after 45 seconds more, but 40 cm. applied after another minute, occluded them. The ear was now engorged with bright blood.

The animal was replaced on its belly and 4 minutes later a pressure of 20 cm. sufficed to shut off the veins. Repeated readings at short intervals yielded the same result, and the arterial pressure, taken next, proved to be 54 cm. with pulsatile flow at 52 cm. A minute afterwards the venous pressure was between 23 and 25 cm.; and finally, after 2 minutes more, the arterial pressure was once again 54 cm.  $H_2O$ .

The distribution of dyes was followed in some of the suspended animals. Controls, of identical weight, anesthetized in the same way but lying on the abdomen, were injected simultaneously. When the animals had been suspended for a few minutes only, the character of the staining showed that the gradient of vascular permeability still existed; but the coloration developed sooner and was much more

intense than in the controls, dye escaping from all of the capillary meshes except those immediately next the arterioles. In addition a zone of stain formed just outside the larger veins. The rapidity with which the dye was carried through the vascular web showed, as had the color of the ear prior to staining, that an active hyperemia was present.

Some mice were suspended for 3 to 4 hours before receiving the dye. The position was well tolerated, but in some cases a slight edema of the ear had developed at the time of injection. At the end of the preliminary period the fan veins had widened markedly, and so too with the lesser veins and venules, the changes persisting for an hour or more after the animals were again prone, as did also the vascular engorgement and high venous pressure. Dye injection while the animal was still suspended caused a rapid, generalized staining, with a broad zone of deeper color along the large fan veins. The fact could still be discerned however, that the staining was progressively more intense along the capillary web and greatest in the tissue about the venules. It was plain that a gradient of permeability still existed along the capillaries, reaching its peak in the venules.

#### DISCUSSION

Under normal circumstances a mounting gradient of permeability exists along the further portion of the capillaries supplying the corium; but the venules into which they empty are more permeable still (1). The present experiments prove that slight increases in venous pressure increase the opportunity for the passage outwards of dye substances from the venules and the further portion of the capillary web without essentially modifying the conditions elsewhere. Greater increases have the added effect of causing the capillary wall further back toward the arteriole to become unusually permeable. Since there is some attendant dilatation of the capillaries one cannot be certain whether the more abundant escape of dye is due to a graded increase in the amount of surface through which diffusion can occur, with some increase in local permeability due to thinning of the wall, or whether the heightened hydrostatic pressure has caused active filtration. Perhaps all these influences are at work. The wall of the larger veins is certainly rendered more permeable by the pressure, for it lets through materials which ordinarily do not pass. Nevertheless the venules remain the most permeable of all the small vessels. Only when the venous pressure is raised nearly to that in the arteries,

and the capillaries, as result, have been forcibly distended, does the characteristic gradient of vascular permeability disappear.

In mammalian skin, especially that of human beings, venules largely take the place of capillaries; and they are differentiated for special functions (9). In voluntary muscle on the other hand the arrangement of the venules, transverse to the muscle fibres, indicates, like their shape and size, that they are merely drainage channels. In muscle the vascular permeability is greatest toward the end of the capillaries, and it is here that a heightened venous pressure exerts its greatest effect, not in the region of the venules (10).

The dyes employed for the observations do not at once become fixed upon, or stored in, the skin but color it because contained in the intercellular fluid into which they pass from the plasma through the barrier of the vessel wall (10). The point is an important one in the present relation because venous pressures which suffice to extend and emphasize markedly the gradient of vascular permeability give rise to edema at the same time. There can be no doubt that the region of greatest escape, under such circumstances, of dyes dissolved in the plasma will also be that of greatest fluid escape.<sup>1</sup> One is justified in inferring from the color pattern that transudation through the small venules is more important for the rapid development of edema of the skin as result of increased venous pressure than is transudation through the capillaries. Edema occurring as the result of vascular injury by heat or cold, on the other hand, comes about mainly by loss of fluid from the capillaries as shown in an accompanying paper (11). Several explanations of the edema of heart disease have been offered in the past (12-15). Not only are the small vessels caught, so to speak, between the arterial pressure and an abnormally high venous one, but nutritive or toxic disturbances of the vascular endothelium may occur and affect permeability. The endothelium of the venules should suffer as much from these problematic disturbances as that of the capillaries, if not more. Most of the edema fluid accumulates in the subcutaneous tissue. Whether it finds its way there secondarily from

<sup>1</sup> This is not to say that wherever dyes escape from the blood under ordinary circumstances there must be a flow of water as well. They pass out by diffusion, in the absence of hydrostatic pressure, and yield the color pattern indicative of the ordinary gradient of vascular permeability (10).



the skin or is the result of fluid escape from the relatively infrequent subcutaneous vessels is a problem as yet unsolved.

The abnormal permeability of large veins widened by high pressure (as evidenced by the escape of dyes into the ears of mice suspended for long periods head down) needs no comment. One may recall in connection with it the seepage of fluid from the abnormally distended veins of human beings.

The capillaries in the soft tissue of the mouse ear transmit pressure with but little loss, as shown by the rapidity with which this mounts behind an obstacle to venous outflow. Our observations confirm those of Landis (16) who found by direct determinations on human capillaries that temporary decreases in the venous flow (as in Valsalva's experiment) cause a prompt intracapillary rise. As he points out, the capillaries that he punctured for the purpose of pressure readings were supported by the firm tissue of the nail-bed and their walls were relatively rigid. This was not the case in the mouse ear. In the mouse ear the least increase in venous pressure that enhances the permeability of the venules, as shown by the rate of escape of dyes, causes also a perceptible widening of the capillaries; but the gradient of permeability along these latter is almost unaffected. High pressures do away with the gradient completely. The uniformity of the staining that develops when the capillary barrier has been broken down by such pressures attests to the fact that the color pattern developing under ordinary circumstances is not due to structural differences in the tissue surrounding the capillaries. Previous work from this laboratory has ruled out the possibility that it is the result of a graded tonic contraction of these vessels or other functional conditions (10). There is good reason to refer it to a structural differentiation along the capillary.

#### SUMMARY

The gradient of permeability which exists along the cutaneous capillaries and venules is accentuated and broadened in scope by increasing the venous pressure moderately. Under such circumstances transudation leading to edema takes place most abundantly from the venules. The permeability of the portion of the capillary web that is near the arterioles increases only when the venous pressure rises so

high as to approximate that in the arteries. Under such circumstances the gradient of permeability along the small vessels disappears, the capillaries and venules everywhere leaking fluid. The character of the vital staining developing under such circumstances indicates, like the evidence of previous work, that the cause for the gradient is to be sought in a structural differentiation.

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#### EXPLANATION OF PLATE 22

FIG. 1. Ears of a mouse after an intravenous injection of pontamine sky blue,—to illustrate some of the changes in permeability when the venous pressure has been raised to a moderate degree. A pressure of 9.4 cm. water was exerted upon the fan vessels of the left ear by means of the apparatus described in the text. The pressure was not great enough to occlude the large veins but compressed them to about half their original diameter and there was an abundant rapid flow past the obstruction. After exerting the pressure for 3 minutes the injection of dye into the circulation was begun, the total quantity being given in half a minute.  $3\frac{1}{2}$  minutes after the end of the injection the pressure was relaxed and the ears were immediately severed and photographed during the next 2 minutes.

It will be seen that in both ears there was a patchy staining. In the control this was slight and it was restricted to the region supplied by the venules and the

furthest portion of the capillary web. In the ear subjected to venous hyperemia the coloration was intense. The dye had escaped in great abundance from the venules and back along the capillaries as well. Some had got out even from the large veins.



1



Photographed by Louis Schmidt



## THE GRADIENT OF PERMEABILITY OF THE SKIN VESSELS AS INFLUENCED BY HEAT, COLD, AND LIGHT

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### PLATE 23

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The object of the work here reported has been to determine whether the gradient of vascular permeability demonstrable in the skin of mammals (1) undergoes alteration during active hyperemia, and to learn the consequences to it of vascular injury by heat and cold. The ear of the mouse has been utilized because any alterations of the gradient of permeability during the distribution of vital dyes from the blood is almost diagrammatically visible in an altered color pattern.

### *General Method*

Young mice of 18 to 20 gm. under luminal anesthesia have been employed throughout. After exposure of one of the ears to heat, cold, or light, a dye was injected into a tail vein, and soon thereafter both ears were lopped off with scissors to check the progress of the staining in them. They were at once arranged symmetrically in paraffin oil under the same large cover glass and studied over white porcelain in a combination of transmitted and reflected light. The exposed ear was always cut off first, since it tended to stain the more promptly. In the amputated organ the vascular network was not as sharply outlined as in the living animal, because of the loss of some of the contents of the vessels, but it could still be seen plainly enough for the relations of the staining to arterioles, capillaries, and venules, respectively, to be readily made out.

Pontamine sky blue, a dye that escapes but slowly, was employed as routine because it serves better than more rapidly escaping materials to disclose slight local differences in vascular permeability. For corroboratory tests Chicago blue 6B was injected. A study of photographs taken at once, after the amputation of ears into which pontamine blue had emerged, and 5 minutes later, disclosed no significant extravascular redistribution of the dye. Nevertheless, the comparison of the experimental and control ears was always carried out very rapidly. The technic of the lighting has been described in a previous paper (1).

*Evidence of the Normal Gradient of Vascular Permeability*

Ordinarily pontamine blue, Chicago blue, and other vital dyes escape with greatest ease through the walls of the small venules of the corium, and a little less readily through the wall of the adjoining portion of the capillary web, the permeability of the capillaries diminishing greatly in the direction of the arterioles. The influence of these local differences is manifest in the color pattern seen in the ear soon after the dye has been put in circulation. Brilliant patches develop in the region supplied from the venules and the further capillary region when as yet no staining has occurred anywhere else in the ear. The restricted distribution of the dye is not referable to specific affinities for certain sorts of tissue. The tissue indeed is not stained, the dye being contained in the interstitial fluid (2). After a greater or less time, depending on the diffusibility of the dye, the colored patches are lost in a general staining which is partly the result of dye escape from the proximal capillary region, and partly due to a secondary spread through the tissues from the region first stained. Any deviation from the general course of events implies an alteration in the opportunity for materials to pass out of the blood.

*The Effects of Heat*

The effects of heat on the color pattern were studied in detail.

The anesthetized mouse was placed on its back upon a cork platform beyond which its head extended over two converging test-tubes about 15 cm. long and 2.5 cm. in diameter into which the ears dipped. The edges of the tubes, which were not flared, came within 1 mm. of each other, and one tube had been filled brim full of water at room temperature, while the other contained water that was kept at the desired warmth by means of a small Bunsen flame. The ears were moistened with alcohol to facilitate immersion, and the hair between them on the top of the head was oiled. Under such conditions the tubes remained full after immersion of the ears, even to a bulging of the meniscus, whereas in lack of the oiling, water was drawn out by capillarity and a drip ensued. The head was held free of the tubes, by a clamp on the skin of the lower lip. When the apparatus had been properly arranged both ears were symmetrically immersed nearly to their bases and they did not touch the glass anywhere. The temperature was followed with a thermometer placed immediately next them. After one ear had been heated for a greater or less time, dye was injected into a tail vein of the mouse without altering its position. As routine, 0.1 cc. of isotonic half strength pontamine blue (a 21.6

per cent watery solution of our new preparation of the dye, mixed with an equal part of Locke's solution) was injected in the course of 1 minute. Isotonic half strength Chicago blue 6B (a 17.1 per cent watery solution mixed equally with Locke's solution) was sometimes substituted. The progress of the staining of the ears was followed through a lens, and when the moment for closer inspection seemed to have arrived the animal was lifted away by means of the mosquito forceps and the ears cut off.

Exposure to water at between 42° and 43°C. for 7 minutes caused a pronounced active hyperemia, the ear becoming bright pink; and staining occurred so rapidly as to necessitate amputation of the organ within less than a minute after the end of the dye injection. It was already thickly patched with dark blue on a paler blue ground (Fig. 1.). Very little dye had as yet left the vessels of the control ear. The patches in the heated specimen were far more numerous than ordinarily, as could be seen when the animal was not killed until patching had begun in the control ear; but they were localized to the tissue about the smallest venular trees, just as usual. Seen amidst the general staining they appeared smaller than ordinary. The capillaries were still visible in the amputated specimen because of dye-stained blood within them, as was not the case in the control.

When the heating at 44°C. had been kept up for 14 minutes prior to dye injection, stain escaped abundantly from all of the small vessels, but still in greatest amount from the venules. The tissue had become slightly edematous prior to the injection (Fig. 2). Exposure to 45°C. for 14 minutes caused a more considerable edema. There now developed a deep diffuse staining with but the slightest intensification in the perivenular region and this soon lost (Fig. 3). At 46°C. for 12 minutes the aural muscles contracted, crinkling the ear. Since a circulatory disturbance due to such cause could not be ruled out, the specimens were discarded.

For additional tests the ear was heated by blowing a fine jet of compressed air through the upper part of a Bunsen flame to reach the ear at a distance of 10 to 15 cm. The mouse was laid on its side upon a dais immediately behind a wooden block above which the outer two-thirds of the ear projected; and the head was so placed at the edge of the dais that the control ear and the region about it were not pressed upon. The temperature was taken with a thermometer moved here and there immediately about the ear, throughout the period of exposure.



The ear in the blast of air soon became brightly hyperemic. Heating at 46°C. for 3 minutes, with dye injection during another minute while the exposure was continued, yielded, in yet another minute, a diffuse staining that was deep and even near the edge of the ear, with an intense patching superimposed on somewhat lighter staining further in toward the head, where the tissue was thicker and the vascular disturbance not so great. Patching had just begun at this time in the control ear,—which was not wholly protected from the warm air,—but the patches were relatively few and pale.

Heating at 56°C. for 4 minutes caused an intense local hyperemia with edema and immediate diffuse staining. Toward the base of the ear, where the heating had been less, a brilliant, abundant patching on a background of diffuse color had developed at the time when the ear was lopped off, 3 minutes after the injection of pontamine blue. Lymphatics laden with dye-stained fluid, from the dark blue, edematous tissue further out, coursed through this region. The control ear showed no staining whatever.

A degree of heating which induces active hyperemia without edema brings about several important changes in the staining of the ear. Patching takes place far more quickly than in the control organ; and the patches are more numerous. Also in the case of pontamine blue diffuse coloration develops concurrently with the patching as never happens with this poorly diffusible pigment under normal conditions,—though with highly diffusible dyes it is a regular occurrence (1). Increased blood flow, heightened intracapillary pressure, and the capillary dilatation of active hyperemia are doubtless responsible for the phenomenon.

Heating that suffices to cause slight edema in the hyperemic ear renders the capillary web in the corium everywhere so permeable to pontamine blue and Chicago blue that an intense diffuse staining follows practically at once upon the injection into the blood stream; yet even under such circumstances, with the effective concentration of dye rapidly diminishing through loss to the tissues on the way to the venules, the escape is still greatest from these latter, the color about them being definitely more intense than elsewhere. Only when heat has so damaged the vessels as to cause fulminant edema is the distribution from the blood approximately an even one, as evidenced by the intense general staining.

*The Effect of Cold*

The cold to which the mouse ear was exposed ranged from that which induced only an active hyperemia to a temperature that caused prompt freezing.

For most of the tests the ear was placed in a current of cold carbon dioxide mixed with air. Care was taken that the organ should be dry, for wet mouse skin lets carbon dioxide through so readily that the tissue pH undergoes alteration in the direction of acidity (3). It was found that when a single large piece of solid carbon dioxide is placed in a funnel held vertically in a ring-stand, and inclosed by inverting upon it another slightly smaller funnel, a continuous jet of cool air emerges from the end of the lower funnel so forcibly as to make itself felt 4 to 5 cm. away. This only happens if the end of the upper funnel is open, for the carbon dioxide evaporates very slowly when no air current can pass through the apparatus.

The anesthetized mouse was kept warm while one ear was exposed to the jet. The width of the latter was conditioned, of course, by the funnel opening. It was wide enough for a thermometer to be put next the ear in the cold stream. Complete precision in the degree of cooling was not necessary to the work.

Ears chilled at 1-4°C. for 10 minutes became brightly hyperemic during this period; and on the injection of dye the characteristic patchy staining took place but much more rapidly than in the control, irrespective of whether cooling was continued or not. This happened even when the hyperemia had largely subsided after removal of the ear from the stream. There was also some diffuse staining at a time when none had taken place in the control.

When the ear was placed close to the end of the funnel, where the temperature of the jet was -2°C., it froze within 1 minute. Immediately that this happened it was taken out to thaw; and dye was injected during the subsequent period of intense hyperemia. The ear stained practically at once, with intense perivenular patchings on a ground of diffuse blue, being already deeply colored at a time when the control was practically unstained.

When the outer two-thirds of the ear was frozen at a low temperature and left in this condition for several minutes, the muscle of the affected part contracted on thawing; and though this contraction wore off and the tissue became bright pink, the circulation was imperfect, as appeared when pontamine blue was injected, the dye penetrating with difficulty or not at all into the region that had been frozen. Nearer the base of the ear was a marginal zone which had become markedly edematous and stained rapidly and diffusely, while still nearer the head,

where there was no edema, an intense, abundant patching of the usual distribution in relation to the vessels was to be seen. The staining took place before any occurred in the control ear.

To find whether carbon dioxide, as such, had any part in the results, the ears of some animals were chilled or frozen in cold alcohol or paraffin oil. The results were identical with those described. When the temperature was so chosen as to produce hyperemia merely, a patching with blue on a ground of lighter color developed.

The findings in ears rendered actively hyperemic by cold, without evident damage to the vessels, resembled those when the hyperemia had been caused by heat; and those when the ears had been sufficiently injured for the development of edema, were like those during heat edema.

### *The Effect of Light*

The effect of light was tested by varying slightly the procedure used for heating the ears.

The mouse was placed on its back, the head held horizontally by a clamp attached to the lip, and the ears immersed in two Petri dishes brim full of water. Light from an arc lamp was cooled by passage through two filters containing Mohr's solution and concentrated to a disc about 6 mm. in diameter upon the submerged part of one of the ears. The other was completely protected by a strip of black cardboard placed between the Petri dishes. A thermometer bulb was submerged next the lighted ear. Throughout the period of experiment the temperature of the water did not rise.

The light, though intense, produced hyperemia but slowly and it was rather closely limited to the illumined part of the ear. When, after nearly an hour, dye was injected, there developed an abundant blue patching of the exposed tissue, with some diffuse staining as well, all this at a time when no coloration had taken place in the control ear. This result was obtained irrespective of whether the light struck on the upper or under surface of the organ.

The findings were like those after a heating or cooling sufficient to produce hyperemia of the ear without edema.

### DISCUSSION

The staining phenomena observed in ears rendered hyperemic by heat, cold, and light were practically identical, as was to have been expected from the fact that "mechanical, electrical, thermal and chemical stimuli all produce essentially the same response of the vessels

of the skin" (4). Dye got out into the exposed tissue much sooner than into the control, and a generalized staining with the poorly diffusible pontamine blue, as result of an escape everywhere along the capillary web, took place simultaneously with a greater staining from the venules, not, as ordinarily, long after venous escape; but save in this respect, and in the unusually abundant patchings with color, the staining pattern was unaltered. Obvious reasons for the observed differences can be found in the quickened local circulation of blood, in the general capillary dilatation, in a circulation through regions where it is not maintained ordinarily, and in the increased pressure prevailing within capillaries and venules. Under normal circumstances there are many regions in the skin of the ear of the rabbit, cat, and dog through which blood flows almost not at all. The same has been found true of the mouse (1). After a dye injection into this animal one observes not a few venules in normal ears containing almost unstained blood, the reason being that none has got through most of the capillaries of the web that they drain; and needless to say, the tissue surrounding such venules remains unstained. Under the circumstances of general capillary relaxation induced by heat, the dye reaches these venules in abundance and staining occurs. Landis has found that the maximal relaxation of the cutaneous vessels of man, produced by heating, causes the pressure in the arteriolar capillaries to double and in the venous capillaries almost to quadruple (5).

To break down the gradient of distribution disclosed by the patchings with stain—a gradient referable in large part at least to a special permeability of the venules and further capillary meshes—it was necessary to produce such injury to the vessel wall as would result in rapid, abundant edema. Only under such circumstances did an approximately even staining of the tissue take place. In the persistence of the gradient despite lesser but still considerable degrees of vascular disturbance one can find a new reason besides those already advanced (1, 6–9) for the assumption that the gradient depends upon the structure of the vessel wall, not on functional conditions. Its practical disappearance when this wall is badly damaged proves that it cannot be the result of local differences in the extravascular fabric, a possibility brought up in a preceding paper.

The experiments disclosed incidentally some of the functional changes which precede and are doubtless responsible for the structural alterations characteristic of injury by heat and cold. One such change deserves special mention. The slightest freezing of the mouse ear, at the highest temperature at which this can soon be accomplished ( $-2^{\circ}\text{C}$ . in our experience),<sup>1</sup> causes the walls of the large arteries to become readily permeable to dyes (pontamine blue, Chicago blue) which fail to pass through them at all under ordinary circumstances. A zone of deep color soon forms along the outside of the injured vessels. With longer freezing at lower temperatures the arterial leakage is accentuated. Most stuffs carried by the blood are greatly more diffusible than the dyes we used, and the damaged arterial wall must provide but a slight barrier to their escape. The fact is known that after freezing, blood platelets soon collect on the walls of the arteries of the affected part, and that arterial thrombosis is responsible for much of the late damage (11). The media of the arteries undergoes a degeneration. It is plain that a pathological seepage through the vessel wall precedes these changes. Probably it has much to do with them.

#### SUMMARY

The mounting gradient of permeability along the small vessels of the corium is essentially unaltered by active hyperemia produced by heat, cold, or light. Only when the vascular walls are so damaged that rapid leakage ensues, as shown by the development of edema, does the permeability of the capillary web as a whole approximate that of the venules. It is plain that the normal gradient of vascular permeability depends upon the integrity of the vessel wall.

The method of experiment described can be utilized for a study of the functional changes which result in the lesions due to burning and freezing.

<sup>1</sup> Mammalian tissues freeze at  $-0.56^{\circ}$  to  $-0.97^{\circ}\text{C}$ . in the absence of circulation (10). Lewis (4) found that a local temperature of  $-2.2^{\circ}\text{C}$ . will freeze the skin of normal human beings.

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## EXPLANATION OF PLATE 23

For purposes of comparison the ears have been transposed, so that the marginal regions lie next each other. All have been photographed from above.

FIG. 1. One ear of an 18 gm. female mouse under luminal anesthesia was submerged for 7 minutes in water between 42.0° and 42.6°C. During the next minute 0.1 cc. of half strength pontamine sky blue was injected intravenously. 52 seconds later the heated ear already showed a pronounced staining. Both ears were cut off, placed side by side on a porcelain plaque, and covered with paraffin oil. The picture was taken approximately 3 minutes after the amputation. The heated ear shows a pronounced patchy staining with some general coloration as well. Dye has just begun to escape from the venules of the control.

FIG. 2. Results of heating an ear at 42-44°C. for 14 minutes. Same general technic as in the experiment of Fig. 1. There are still local differences in staining indicative of a gradient of vascular permeability. No dye has escaped as yet into the control ear.

FIG. 3. Results of heating an ear at 45°C. for 14 minutes; technic as in the experiments of Figs. 1 and 2. The fine white dotting is caused by the dispersion of light by the sebaceous glands situated around the hair follicles.





1



3

Photographed by Louis Schmidt

(Hudack and McMaster: Gradient of permeability of skin vessels)





## PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

### II. THE INACTIVATION OF THE TUMOR-PRODUCING AGENT BY MONO- CHROMATIC ULTRA-VIOLET LIGHT\*

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Early work on Chicken Tumor I indicated that, while the sarcoma cells are as susceptible to ultra-violet radiation as other cells, the etiological agent separable from the cells is relatively resistant (1). Later Baker and Peacock (2) estimated that five times the lethal dose for pathogenic bacteria was not sufficient to destroy the activity of the chicken tumor agent, and that even eight times the amount did not invariably destroy the activity (7). This observation was confirmed in general by Illingworth and Alexander (3). All these observations were made without particular reference to wave length or the absolute energy involved. One of the present authors (Gates (4)), using measured monochromatic light and a standard technique, has been engaged in a comparative study of the energies required to kill or inactivate various organisms or biological agents at single wave lengths in the ultra-violet region. By plotting the energies required against the corresponding wave lengths, similar graphs are obtained for comparing the qualitative and quantitative action of ultra-violet light. This method offers an opportunity to compare the reaction of the tumor agent with that of bacterial cells, virus or phage.<sup>1</sup>

\* This investigation was carried out by means of funds from the Rutherford Donation.

<sup>1</sup> A preliminary report on this work was published in the *Internat. Conf. Cancer*, London, 1928, 33.

### *Method*

1 gm. of finely mashed Chicken Tumor I, or an equal amount of tumor desiccate, was emulsified with 10 cc. of water, thoroughly shaken, centrifuged at high speed, and the supernatant fluid passed through a filter paper. The bottom surface of a small Petri plate (4.5 cm. in diameter) was covered with enough melted agar to form a layer 1.5 to 2 mm. thick after solidification.<sup>2</sup> On this smooth surface 2 drops of the thick tumor filtrate was evenly spread and allowed to stand at room temperature for about 45 minutes, when sufficient drying had occurred to prevent flowing of the material. Uniform strips, 3 x 20 mm., were then cut from the middle of the agar plate and exposed to varying doses of ultra-violet radiation. With a quartz mercury arc as the source of energy, the specimens were placed behind the exit slit of a large monochromatic illuminator for various intervals of time. The wave lengths selected lay between  $\lambda$  238 and  $\lambda$  313 m $\mu$ , and the intensity of the radiation at each wave length (measured in ergs per mm.<sup>2</sup> sec. by means of a standardized thermopile and high sensitivity galvanometer) multiplied by the time of exposure gave the total energy per mm.<sup>2</sup> for each exposure. Immediately afterwards the strips were loaded in 16 gauge lumbar puncture needles and injected intradermally in chickens, each chicken receiving also a control unexposed strip which had been kept under the same general conditions as the test specimens.

The uniformity of the control "takes" and the reasonable regularity of results with the exposed specimens indicated that the presence of the neutral agar had no significant effect on the reactions.

### RESULTS

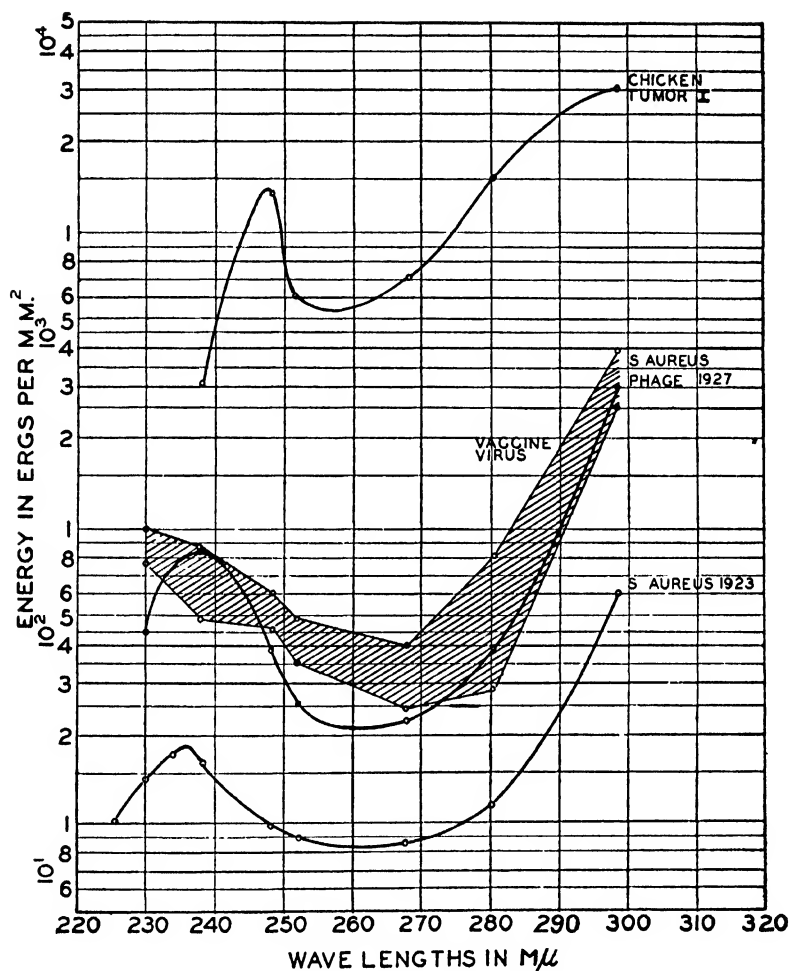
The results of 624 irradiation tests are shown graphically in Text-fig. 1. The curve is based on the points at which the agent was attenuated to such an extent that tumors resulted from less than 50 per cent of the test inoculations. The results for  $\lambda$  313 m $\mu$  are not shown, as the tumor agent was not inactivated even by an exposure of 80,000 ergs per mm.<sup>2</sup>

Ultra-violet inactivation curves for a bacterium (4), a typical virus (5) and a phage (6) have been plotted in Text-fig. 1 for comparison.

The energy required at each wave length to inactivate the tumor agent is far greater than that required to kill or inactivate bacteria, virus or phage.<sup>3</sup> It is of equal importance to note the relative differ-

<sup>2</sup> In preliminary experiments carried out to test the suitability of this method, gelatine was used, but as it was found that this substance had a definite enhancing action on the tumor agent, agar was substituted.

<sup>3</sup> Baker and Nanavutty, working with an unresolved ultra-violet spectrum, estimate that phage has the same degree of susceptibility as bacteria, that the chicken



TEXT-FIG. 1. The points on the curves drawn for *S. aureus*, *S. aureus* phage and Chicken Tumor I represent the energies required to reduce the subsequent colony or plaque formation or tumor takes to 50 per cent of those obtained with control specimens.

The cross-hatched area shows the limits of energy in various experiments which resulted in the failure of exposed vaccine virus to produce lesions in susceptible rabbits.

tumor is 8 times more resistant, while ferments and antibodies are 20 to 120 times more resistant (7).

ences in activity of the various wave lengths examined. The general form of the curves for bacteria, virus and phage is similar. Contrasting these with the curve for the tumor agent, it is seen that among the shorter wave lengths tested the most active for the tumor agent ( $\lambda 238$ ) is least active for the other group; and the least active for the tumor agent ( $\lambda 248$ ) is in the range of the most active wave lengths for bacteria, virus and phage.

#### DISCUSSION AND SUMMARY

Even though part of the energy of the incident light is probably absorbed by chemical entities which play no part in the specific reaction of inactivation, nevertheless the wave lengths most active in destroying biological cells or agents will presumably be found to be among those absorbed in the highest proportion. This would indicate that the curves here presented are approximately reciprocal to the coefficients of absorption of particular substances, the destruction of which caused the inactivation of the agents or the death of the cells. The similarity between the curves for bacteria, virus, and phage, both in shape and in total involved energies, suggests the presence of a common factor, or of closely related chemical entities, sensitive to ultra-violet light, whereas the data for the tumor agent suggest that its inactivation is due to the destruction of a substance having an essentially different spectral absorption, and therefore of a different chemical character. While the amount of ultra-violet energy required to affect the tumor agent is great, it is still less than that involved in the inactivation of some of the enzymes (7).

A study is under way to compare the deduced spectral analysis with the actual coefficients of absorption of the highly purified tumor agent.

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## THE ASSOCIATION OF PNEUMOCOCCI, HEMOPHILUS INFLUENZAE, AND STREPTOCOCCUS HEMOLYTICUS WITH CORYZA, PHARYNGITIS, AND SINUSITIS IN MAN

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In a previous paper (1) it was reported that persons harboring pneumococci in their noses and throats were relatively subject to coryza, pharyngitis, and sinusitis, and conversely that persons not harboring these organisms were relatively free of these diseases. Moreover, it was stated that the occurrence and degree of symptoms were associated to some extent with variations in the numbers of pneumococci obtained from the nose and throat cultures. These relationships are described in more detail in the present paper.

### *Technique*

The general scheme of study has been described previously (1). The group on which the observations were made comprised adults working at The Rockefeller Institute and in some instances their children. Each individual was questioned every 2 or 3 days to ascertain the presence of fever, chill, malaise, headache, nasal discharge, sore throat, sneezing, cough, etc., and was requested to report whenever such symptoms occurred. Cultures of the nasal passages and throat were usually made weekly; when symptoms were present, however, cultures were taken daily, if possible. Material obtained by means of a sterile swab was streaked over one or two freshly prepared 15 cm. rabbit blood agar plates. The resultant aerobic growth was classified into Gram-positive and negative cocci and bacilli. Pneumococci, *S. hemolyticus*, and *H. influenzae* were identified with care (1).

### RESULTS

The present analysis is based on the protocols of the individuals studied most intensively. Since considerable information about each case is needed in order to judge the degree of association of upper respiratory tract symptoms and potential pathogens, the selected

group constitutes less than 20 per cent of the entire number studied. This association, moreover, became increasingly apparent with the amount of care exercised in obtaining full histories, in taking frequent cultures, and examining them minutely.

The protocols are divided into those of individuals not carriers of pneumococci, *H. influenzae*, and *S. hemolyticus*, and individuals who were transient, periodic, and chronic carriers of these organisms. Illustrative cases are described in the following paragraphs.

*Non-Carriers.*—Two in number. Were free of symptoms 2½ years and 1 year respectively.

*Transient Carriers.*—

Case 1, free of upper respiratory tract symptoms and pathogens on 25 tests from Sept. 4, 1928, to Nov. 27, 1928, suffered an influenzal attack from Nov. 26, 1928, to Dec. 10, 1928, during the epidemic. At this time, 6 of 32 cultures contained large amounts of *H. influenzae* and 2 contained pneumococci of an unnumbered, specific type. Subsequently, tests were negative and remained so during a 9 day period of sore throat from Feb. 1, 1929, to Feb. 10, 1929. Thereafter no symptoms were reported and 34 cultures contained none of the above mentioned organisms. On Nov. 17, 1930, the individual reported sore throat lasting 24 hours and yielded *H. influenzae* on this date and 1 week later. The succeeding 17 cultures were free of these organisms and no further symptoms were noted until Apr. 20, 1931, at which time chills, weakness, sneezing, nasal discharge, and sore throat were experienced for 2 days and *H. influenzae* was recovered on Apr. 20, 1931. 7 tests thereafter were negative; 1 on Aug. 27, 1931, contained *H. influenzae*; 5 tests then proved negative. On Oct. 19, 1931, chills, fever, malaise, and sore throat developed, lasting 5 days. During this time and thereafter to Nov. 1, 1931, 5 cultures were taken, all negative for the organisms.

Case 55 was a carrier of pneumococci Type XIII on 14 tests from Oct. 11, 1929, to Feb. 7, 1930. During this time one 6 day period of nasal discharge preceded by malaise, nasal obstruction, and sore throat was noted. Tests were negative from Feb. 7 to Apr. 15, 1930. From Apr. 11 to Apr. 22, 1930, the individual suffered a relatively severe attack of prostration, irritability, and general aching sensations, accompanied by sneezing, nasal obstruction, and slight discharge. On Apr. 15 and 16, *S. hemolyticus* was recovered from the throat. Subsequent tests were negative. On Oct. 14, 1931, malaise and nasal congestion were reported, but 5 tests from Sept. 24 to Oct. 27, 1931, were negative. Commencing Oct. 30, 1931, a moderately severe attack of coryza, lasting 8 days, was experienced, and 2 weeks later, a second attack, lasting 10 days, was reported. 7 of 9 cultures taken during this period contained *S. hemolyticus*. Shortly after the symptoms disappeared, the tests became negative and remained so for 2½ months, during which time a 48 hour period of nasal discharge and sore throat







occurred. On Feb. 13, sore throat and nasal discharge were reported, lasting 5 days, and on Feb. 14 and 16 *H. influenzae* was obtained. No further symptoms were reported and subsequent tests were negative for these organisms<sup>1</sup> (Text-fig. 1).

These cases are examples of individuals who, on brief and infrequent occasions, carry a few pneumococci, *H. influenzae*, or *S. hemolyticus*. Their appearance is usually associated with the presence of upper respiratory tract symptoms, although symptoms may occur during a period of negative cultures. The organisms usually decrease in numbers or disappear at varying intervals after the symptoms have subsided.

*Periodic Carriers.*—

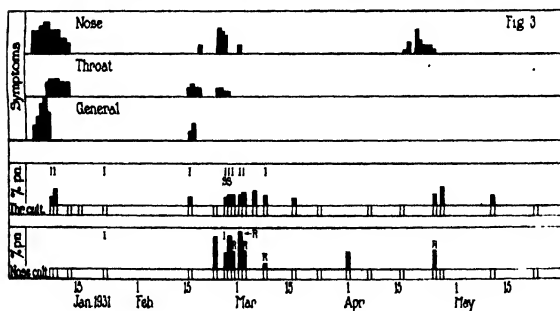
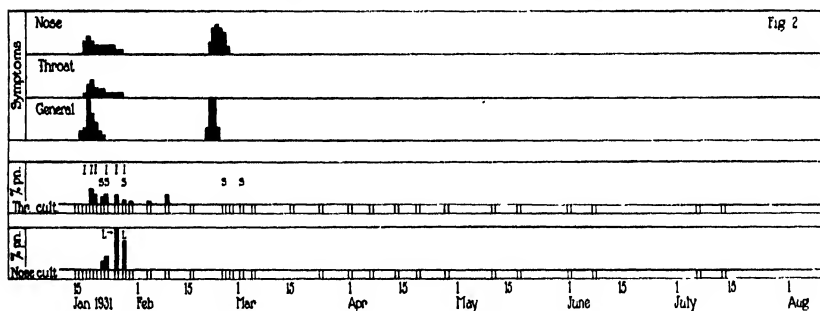
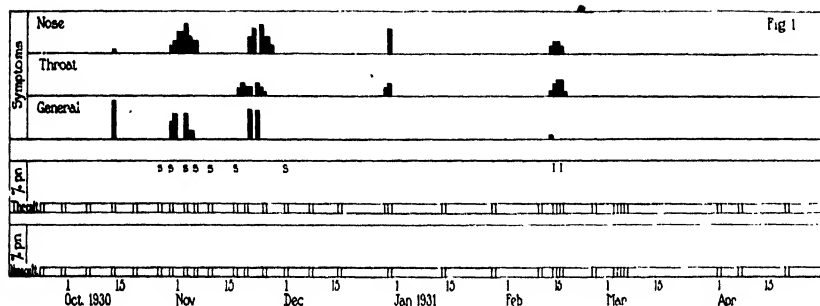
Case 17, free of pneumococci on 34 tests, from January, 1929, to Jan. 17, 1931, and free of upper respiratory symptoms, save for one 6 day attack of coryza and cough which was not studied, developed on Jan. 16, 1931, chill, nasal discharge, sore throat, and cough which persisted 11 days. Cultures taken the day after the onset of symptoms were negative; those taken daily thereafter, to Jan. 30 and Feb. 4 and 9, contained abundant pneumococci of a single, unnamed type, together with *H. influenzae* and *S. hemolyticus*. A second similar attack was reported from Feb. 20, 1931, to Feb. 24, 1931, but only 1 test was made and this proved to be negative. Thereafter, no further symptoms were noted and 21 tests were negative (Text-fig. 2).

Case 50, free of the organisms on 8 cultures over a 5 months' period, developed on Mar. 3, 1931, chills, malaise, headache, and sore throat, lasting 72 hours. Tests Mar. 30, 1931, Apr. 1, Apr. 7, and Apr. 14 revealed pneumococci Types VIII and XXII and *H. influenzae*. 10 subsequent cultures were free of these organisms and no further symptoms have been reported.

Case 51, suffering a severe attack of fever, chills, malaise, nasal obstruction and discharge, sore throat, and cough from Jan. 3, 1931, to Jan. 13, 1931, was first cultured Jan. 8 and 9. Pneumococcus Type V and *H. influenzae* were present. A second attack, with nasal discharge and sore throat, lasted from Feb. 16, 1931, to Mar. 8, 1931, during which time 6 cultures were taken, and was accompanied by the presence of Pneumococcus Type X, *H. influenzae*, and occasional *S. hemolyticus*. During a third 7 day period of coryza, 11 cultures were taken which contained these same organisms. 4 subsequent tests were negative, with the exception of 1 instance, when pneumococci Type X appeared in the culture from

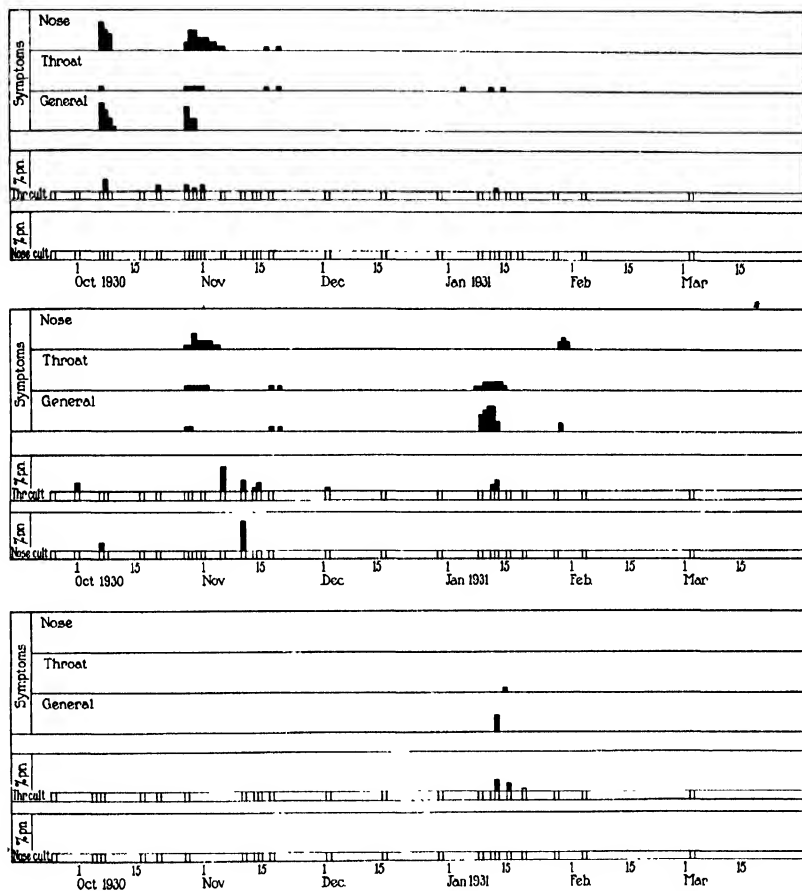
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<sup>1</sup> During the past 17 weeks, Oct. 15, 1931, to time of writing, typical meningococci Type II have been recovered in large numbers and it is not known how long these organisms had been present but unrecognized.



1 = *Hem influenzae*  
 S = *S. hemolyticus*  
 L = Left naris  
 R = Right  
 7 pn = Per cent pneumococci on plate

TEXT-FIGS. 1, 2, and 3



TEXT-FIG. 4

the right naris. From Apr. 17, 1931, to Apr. 25, nasal congestion and discharge were reported; on Apr. 25 and 27 and May 11, *Pneumococcus* Type X was recovered. 1 culture taken May 23, 1931, contained none of these organisms (Text-fig. 3).

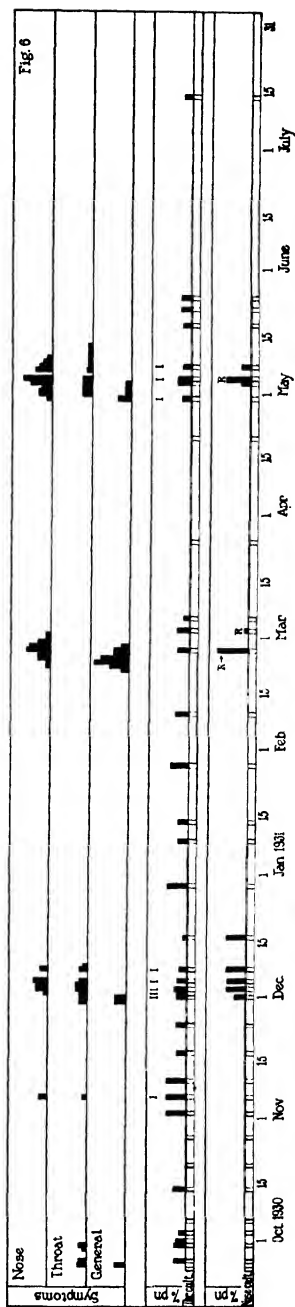
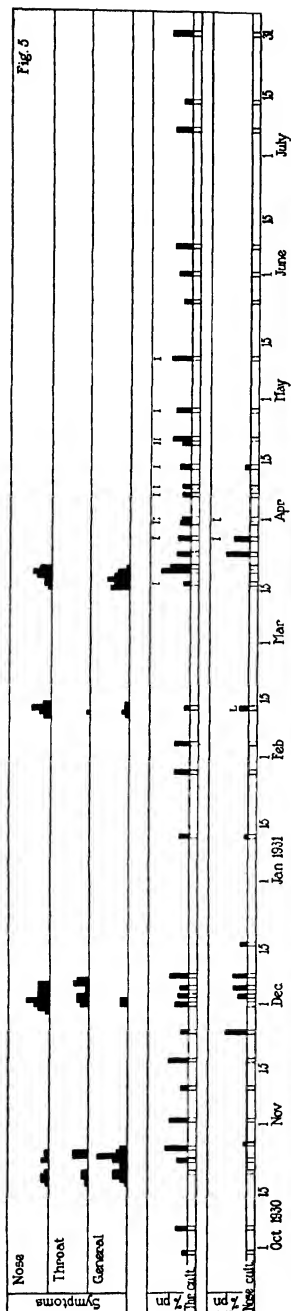
Cases 33, 34, 35, and 36 are members of a family. The father, No. 36, has been free of pneumococcus with the exception of 1 brief period, and free of upper respiratory tract symptoms for 3 years. The mother, No. 35, has been a transient carrier of pneumococci on 3 occasions, during the past 3 years, associated with the appearance of upper respiratory tract symptoms. The 2 children, Nos. 33 and 34, have carried the same type of pneumococci during 3 winter periods and have experienced 2 or 3 mild attacks of coryza and cough. The present study was made during the winter of 1930-1931. One child, No. 33, after 2 negative cultures in September, became a carrier of pneumococci of a specific, unnumbered type on Oct. 10, 1930. The other child, No. 34, after 3 negative cultures, suffered a mild 3 day attack of coryza, during which pneumococci of the same type appeared on culture. Subsequently, both children continued to carry this organism on 21 cultures for the remainder of the winter. They experienced simultaneous attacks of coryza and cough for 2 to 10 days on 3 separate occasions. During the most severe attack, the mother, No. 35, came down with malaise and sore throat, and yielded pneumococci of the same type as found in the children. Thereafter, tests became negative and no symptoms were noted until October, 1931 (Text-fig. 4).

In this group of individuals, the organisms appeared during illness and disappeared at varying intervals after the symptoms ceased.

#### *Chronic Carriers.*—

Case 15 has been a carrier of *Pneumococcus* Type XIII for at least 4 years and Case 52 a carrier of an unnumbered specific type of pneumococcus for at least 1 year. Each has experienced during the winter months 4 to 6 attacks of frontal headache, malaise, nasal discharge, and sore throat. On these occasions, roentgenographs have shown extensive clouding of the antra, cultures from the throat show a great increase in numbers of organisms, and cultures from the nares, previously free, show the organisms present often in nearly pure culture. Moreover, in instances when the individual's pain was localized to one antrum, this localization corresponded to the localization of clouding in the roentgenogram and to the localization of the organisms in right or left nares. When symptoms subsided, the nasal cultures became free of pneumococci, the throat cultures contained them in fewer numbers, and the roentgenographs showed some clearing of the antra (Text-figs. 5 and 6).

In brief, chronic carriers of pneumococci proved subject to respiratory tract symptoms and antrum disease. There appeared, moreover, a direct relation between the degree of symptoms, amount of



TEXT-FIGS. 5 and 6

clouding of antra in roentgenographs, and numbers of organisms contained on throat and nose cultures.

A relationship between the presence of these pathogens and symptoms, although demonstrated in all cases of this special series, may not invariably exist. Indeed, in three cases studied with less care during 1928 and 1929, no such organisms were found during attacks of upper respiratory tract disease. The limits of this relationship therefore must be decided by painstaking study.

#### DISCUSSION AND CONCLUSIONS

Pneumococci, *H. influenzae*, and *S. hemolyticus* are known to be frequent inhabitants of the upper respiratory tract, but most workers have not recognized any definite relationships between their presence and coryza, sore throat, influenzal, and sinusitis attacks (2-5). Dochez, Shibley, and Mills, however, in their experimental studies of common cold, state that in both the spontaneous and experimentally induced "colds" in anthropoid apes, the "most significant change observed has been the increase of activity on the part of the potential pathogens habitually present in the throat flora. Coincident with the appearance of symptoms, pneumococci, *S. hemolyticus*, and *B. Pfeifferi* have developed in greatly increased numbers and have spread over a wide area of the nasopharyngeal mucous membranes. These organisms became at this time conspicuous even in the nose, where they are seldom or never present under normal conditions. The same phenomena have not been observed in human beings" (6, 7).

The essential facts of the present observations are that persons free of pneumococci, *H. influenzae*, and *S. hemolyticus* are in general free of coryza, sore throat, influenzal and sinus attacks; that persons who are occasional or periodic carriers of these organisms may be negative on tests over long healthy periods, but generally become positive during or following attacks and subsequently become negative again; finally, that persons who are chronic carriers show during these illnesses increasing numbers of organisms in the throat and extension of the organisms to the nose.

That these organisms may be the actual incitants has been claimed by Park (8); that they are secondary invaders is the view of Shibley,

Mills, and Dochez who state as a result of their experimental work on this subject that "the most important significance of viruses of this type [common cold] seems to lie in their capacity to incite activity on the part of the more dangerous pathogenic organisms that infect the upper respiratory tract" (7). The present observations bring out the intimate relationship between these pathogens and upper respiratory tract symptoms, but do not disclose the nature of this relationship.

Finally, an addition has been made to the knowledge of the mode of spread of these organisms. A focus of growth and dissemination has been determined in the nasal passages and throat of individuals with chronic upper respiratory tract disease and increases in numbers of the organisms at the focus and their spread to contacts have been related to the winter season and to the occurrence of symptoms in the carrier. The observations suggest that the dosage of these organisms in a community is controlled by the resistance of the carrier and of the contacts. This view is in agreement with the facts derived from studies of native animal infections (9).

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## THE EFFECT OF TESTICLE EXTRACT ON THE GROWTH OF TRANSPLANTABLE MOUSE TUMORS\*

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The recent work of Duran-Reynals (1) has demonstrated that simple water extracts of rat and rabbit testicle, when brought in contact with cell suspensions of the Brown-Pearce rabbit tumor, exert a definite inhibiting effect upon the transplantability and growth of cells from such suspensions in the intradermal tissues of rabbits. This action, which can also be produced by an almost protein-free fraction, is in sharp contrast to the recognized power of extracts of testicle and, to a minor degree, of skin and some other organs, to enhance many bacterial and virus infections (2-7). It has seemed desirable to test the action of these organ extracts upon other groups of tumors and to determine more definitely the nature of such activity.

### *The Effect of Testicle Extract on Spontaneous Tumors*

The first material utilized consisted of tumors occurring spontaneously in the strains of mice propagated in this laboratory.

The tumors varied considerably in size and location, but microscopically all fell into the class of simple or papillomatous adenocarcinomata. They were excised under aseptic conditions with as little damage as possible to the host and, after the healthy tumor material was separated from the surrounding tissue and freed from necrotic areas, it was treated in one of two ways. In the first fifteen instances the material was minced, passed through a double layer of sterile gauze, and then halved. One portion was mixed with an equal volume of a Ringer's solution extract of normal rat testicle, and the other with the same amount of plain Ringer's solution as control. Testicle extract was prepared in the following manner: Adult rats were chloroformed, bled, and the testes removed aseptically. The pulp was ground with sand, extracted with Ringer's solution, 1 cc. per gram

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\* This investigation was carried on by means of funds from the Rutherford Donation.

of pulp, and centrifuged. The supernatant fluid, or so called testicle extract, was used within 3 hours.

Both suspensions were incubated at 37°C. for 2 or 2½ hours and reinjected intradermally in corresponding regions of the abdomen or chest wall of the mouse from which the tumor had been removed. The growth of the tumors was measured weekly. The animals died or were killed from 13 to 62 days after injection. The nature of the tumor was confirmed histologically in every case.

In the second group of 20 mice, the procedure was the same as that described above, except that grafts of the tumor tissue about 3 mm. in diameter were used instead of cell suspensions. These were immersed in 1 cc. of testicle extract and,

TABLE I

*Effect of Testicle Extract on Autografts of Spontaneous Tumors*  
*A. Influence on Growth*

Testicle extract plus	Growth of test tumor. No growth of control	Larger growth than control	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
Tumor cell suspension.....	1	1	0	4	2
Tumor graft.....	0	5	4	5	1

*B. Influence on Transplantability*

Type of transplant	No. mice inoculated	No. successful inoculations	Successful inoculations
			<i>per cent</i>
Tumor suspension + Ringer's.....	15	7	47
Tumor suspension + T. E.....	15	6	40
Tumor graft + Ringer's.....	20	15	75
Tumor graft + T. E.....	20	14	70

after 1 hour incubation, were inoculated into the original host. The grafts for control were incubated in Ringer's solution for the same length of time before inoculation.

The results of these two experiments, in which twenty-three of the thirty-five inoculations resulted in tumors, are shown in Table I.

While the treated tumor material gave a smaller number of takes and the tumors grew somewhat more slowly than the controls in some instances, the difference was not of sufficient magnitude to be considered of importance. Histological examination of all tumors failed

to show any difference between the tumors arising from the treated grafts and the controls.

*The Effect of Testicle Extract on Transplanted Tumors*

*Bashford Carcinoma 63.*—The next tests were carried out with a transplantable adenocarcinoma, the Bashford 63.

This tumor of relatively low malignancy was giving at the time of the experiments about 75 per cent of takes on routine transplantation. Preliminary tests showed that the tumor was not easily transplanted by cell suspensions. Therefore, in the twelve experiments, grafts of the tumor about 3 mm. in diameter were

TABLE II  
*Effect of Rat Testicle Extract upon Bashford Carcinoma*  
*A. Influence on Growth Rate*

Type of transplant	No. experiment	No. mice successfully inoculated	Growth of test tumor. No growth of control	Larger growth than control	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
Tumor graft plus testicle extract. . . .	12	59	9	6	16	18	10

*B. Influence on Transplantability*

Type of tumor	No. mice inoculated	Tumor + Ringer's solution			Tumor + testicle extract		
		No. successful inoculations	Success-ful inoc-ulations	Average diam-eter	No. success-ful inoc-ulations	Success-ful inoc-ulations	Average diam-eter
			per cent	cm.		per cent	cm.
Graft.....	74	43	58	1.28	42	57	1.08
Bolting cloth emulsion.....	20	1	5		1	5	

used. Before immersion in testicle extract or Ringer's solution these were cut through in several places so as to give a greater surface of exposure. The test grafts were kept in the extract and the controls in Ringer's solution for 30 to 90 minutes at 37°C., except in two experiments in which they remained at room temperature. The results of inoculation of these materials into 94 mice are shown in Table II.

The percentage of successful transplants from test and control grafts was almost identical, indicating the absence of any demonstrable effect of the testicle extract.

*Mouse Sarcoma S/37.*—A transplantable mouse sarcoma, known as S/37, was used in the next group of experiments.

This tumor regularly gives 100 per cent takes, develops rapidly, and usually ulcerates between the 12th and 16th day. A *brei* was prepared from healthy tumor tissue by passing it through a masher with 1 mm. apertures. Half of the material was placed in testicle extract and the other half in Ringer's solution. The time of contact varied in the different experiments between 30 minutes and 2½ hours. In two of the six experiments the mixtures were kept at 37°C. while the others remained at room temperature. Each of the 48 mice was inoculated with both the tumor tissue after contact with testicle extract and that which had been kept in Ringer's solution.

All of the inoculations resulted in tumors and there was no significant difference in size between the test and the control ones.

*Attenuated S/37.*—It seemed possible that the unusual malignancy of the S/37 tumor might mask a potential inhibiting factor in testicle extract. To test this point advantage was taken of the observation that the growth rate of this tumor is reduced if the grafts are immersed before inoculation in a fluid the pH of which is varied in either direction from the neutral point. In fact the growth rate of grafts treated in this way is inversely proportional to the degree of deviation of the hydrogen ion concentration of the fluid (unpublished observation).

Healthy S/37 tumor tissue was first passed through a masher, then suspended in a few drops of Ringer's solution and squeezed through sterile bolting cloth. By this process the tumor was broken up into small clumps comprised of not more than thirty or forty cells each. The suspensions were divided into two equal portions, one of which was diluted with an equal volume of testicle extract and the other with an equal volume of Ringer's solution. In some of the experiments a Berkefeld filtrate of testicle extract was used. The fluids of the two cell suspensions, testicle extract and Ringer's solution, were adjusted to a pH which had previously been shown to attenuate, without completely suppressing, the growth of the grafts. In part of the experiments the pH was adjusted to 5.2 by the addition of an acetate buffer, and in the others to 8.4 with a phosphate or boric acid buffer. The tumor cells were kept in this adjusted mixture for 75 minutes at room temperature.

The above described procedure was varied in two additional groups of experiments. In the first, the Ringer's solution in which the tumor cells were suspended was adjusted to a pH of 5.2. After about an hour this suspension was divided into two portions, one diluted with an equal amount of testicle extract and the other with Ringer's solution, and then immediately injected into mice. In the second group the growth activity of the tumors was reduced by heating at 45°C. for 5 to

15 minutes, after which the tumor cells were suspended in testicle extract or Ringer's solution for a period before inoculation.

The results of all the experiments with modified S/37 are shown in Table III.

It is evident from the figures in Table III that contact with either a plain testicle extract or its filtrate adversely affected the transplanta-

TABLE III  
*Effect of Testicle Extract on Modified Mouse Sarcoma S/37*

Group	Modification of tumor	Type of testicle extract	No. experiments	No. mice inoculated	Growth of test tumor. No growth of control	Larger growth than control	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
I	pH 8.4	Plain	2	11	0	1	3	7	0
II	pH 5.2	"	6	30	0	3	9	5	13
III	pH 8.2	Berkefeld filtrate	1	6	0	0	0	0	6
IV	pH 5.2	" "	4	23	0	4	2	9	8
V*	pH 5.2	" "	3	14	0	0	4	7	3
Total I-V.....			16	84	0	8	18	28	30
VI	45°C. for 5-15 min.	Plain	4	30	2	0	5	11	12
Total I-VI.....			20	114	2	8	23	39	42

\*In this group the tumor was divided into test and control portions after modification, and contact with testicle extract was reduced to a minimum.

bility and growth rate of the modified tumor. Inasmuch as the mixtures of tumor and testicle extract in some cases required slightly more buffer than the control, one might assume this to be a possible cause of the results. Such is not the case, however, as experiments in Group V show. Here modification of the pH was accomplished before the suspension was divided into test and control portions, thus eliminating variations due to the buffer; yet the inhibiting effect remained. This group furnished an even more stringent test than the others, in that

the period of contact with testicle extract outside of the body covered only the time necessary for inoculation. The results suggest that a very short exposure suffices to produce an inhibitory effect. The action of testicle extract on the tumor attenuated by heat again gave evidence of the inhibitory action.

*Mouse Sarcoma 180.*—It was suggested above that the failure of the testicle extract to influence the growth of the unmodified S/37 was due to its extreme malignancy. That this was probably the correct deduction was indicated by the results with the attenuated tumor. To test this point further the same experimental procedure has been carried out with a mouse sarcoma of a lower grade of malignancy. This tumor, No. 180, grows well, takes in well over 90 per cent of cases, and is

TABLE IV  
*Effect of Testicle Extract on Mouse Sarcoma 180*

Tumor emulsion plus	No. experiments	No. mice inoculated	Growth of test tumor. No growth of control	Larger growth than control	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
Plain testicle extract.....	6	29	2	2	5	15	5
Berkefeld filtrate.....	2	12		2	3	4	3
Total.....	8	41	2	4	8	19	8

principally notable for the fact that the usual methods of induction of immunity fail to influence its growth.

The same technique was used in the preparation of the tumor material and the testicle extract as that described above, except that there was no modification of the pH of the solutions. The suspensions were kept at room temperature for 75 minutes. Each mouse received an injection of both the testicle extract suspension and the control suspension in Ringer's solution.

The results, which show a definite inhibiting action of the testicle extract on Sarcoma 180, are shown in Table IV.

*Effect of Purified Testicle Extract on Mouse Tumors*

The inhibiting action of rat testicle extract on Mouse Sarcoma 180 and the attenuated S/37 is definitely shown by the findings here

reported. Duran-Reynals has reported that a purified fraction of testicle extract inhibits the growth of the Brown-Pearce rabbit tumor in much the same degree as the full extract (1). This fraction<sup>1</sup> is known to contain in concentrated form the so called Reynals factor which increases the intradermal spread of injected materials and enhances the action of infectious agents. The effect of this fraction was next tested on mouse tumors.

TABLE V  
*Effect of Purified Testicle Extract on Sarcoma S/37 and 180*

Type tumor	No. experiments	No. mice inoculated	Larger growth than control	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
S/37	1	7		7		
S/37 pH 5.2	2	14	2	10	2	
180	4	21	6	8	6	1
Total . . . . .	7	42	8	25	8	1

This group of experiments included tests on S/37 and its modified strain as well as on Sarcoma 180. The technique was the same as that described in the foregoing experiments, except that the purified fraction was used instead of the full testicle extract. The results as shown in Table V yield no evidence of augmentation or inhibition of tumor growth.

#### *Effect of Heat on the Inhibiting Property of Testicle Extract*

The "spreading factor" in plain testicle extract is completely inactivated at a temperature of 60°C. (1, 3), though purified extracts retain their activity even after boiling. If increase in tumor cell permeability were the factor causing inhibition of growth, one would expect this property to be lost on heating.

Testicle extract was heated at 60°, 80°, and 100° for 10 minutes, then mixed with tumor emulsions as in previous experiments. As there was no diminution in the inhibiting action of the extracts heated

<sup>1</sup> The details of the purification technique will be published later. The fraction in 0.4 gm. has the enhancement value of 100 gm. of testicle material.



at different temperatures, the results are tabulated together in Table VI. A control group in which muscle extract was used instead of testicle extract showed no inhibiting action. In fact the tumors treated with this material grew somewhat more rapidly than the controls.

TABLE VI  
*Effect of Heated Testicle Extract on Sarcomata S/37 and 180*

Type of tumor	Type of extract	No. inoculated	Same growth as control	Smaller growth than control	No growth of test tumor. Growth of control
180	Heated 60-100°C.	14	3	8	3
Modified S/37	" 60-100° "	12	7	3	2
Total.....		26	10	11	5

#### DISCUSSION

The experiments reported above show that testicle extract will inhibit the growth of a mouse sarcoma of a moderate degree of malignancy and an attenuated strain of another more highly malignant sarcoma. The manner in which the testicle extract exerts its depressant effect on the tumors is not clear. Devitalization of the tumor cells, or the stimulation of resistance in the host, or a combined effect are the possibilities to be considered. There is much evidence that the Reynals factor in testicle extract, which enhances the action of infectious agents, produces its effect at least in part by increasing tissue and cell permeability (4, 8, 9). The fact that the fraction of testicle extract containing this factor in purified form was just as effective as the whole extract in inhibiting the Brown-Pearce rabbit tumor led Reynals to suggest that the tumor inhibiting and infection enhancing influences are manifestations of the action of a single agent. However, in the present experiments there is no evidence that the purified fraction has any effect on the mouse tumors. The question raised by this finding must receive further investigations before a conclusion can be reached.

The fact that the tumor-inhibiting property of testicle extract is not destroyed by heat, while the infection-enhancing power is destroyed, adds another point of difference. However, it has been shown that the Reynals factor freed from much of the inert testicle protein is itself

heat-stable. It may well be that the heat inactivation of the crude extract is due to a temporary adsorption of the factor on the coagulated protein, and that in the experiments with tumors there is a gradual release after the material is inoculated into the body.

The relationship of the Reynals factor to the tumor-inhibiting property of testicle extract is not definitely determined by the present work, nor have we sufficient evidence to indicate that the phenomenon here described is due to the same mechanism as the inhibition of Chicken Tumor I (10).

#### SUMMARY

Grafts of a transplantable mouse sarcoma designated as No. 180, and those of an attenuated strain of a more malignant Sarcoma S/37, treated with testicle extract, either fail to grow on inoculation or result in tumors of a lower growth rate than the controls. Autografts of spontaneous mouse tumors so treated show little if any effect, while the Bashford adenocarcinoma and the unattenuated S/37 are unaffected. The factor in testicle extract responsible for the retarding activity passes readily through a Berkefeld filter and is thermostable.

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## CELLULAR REACTIONS TO FRACTIONS ISOLATED FROM TUBERCLE BACILLI

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The cellular reactions in tuberculosis form one of the striking features of the disease and it is important to note that they can be produced by the dead bacilli. In 1890, Maffucci (1) studied the effect of subcutaneous injections of dead organisms; he recorded the formation of abscesses and the subsequent death of the animals from marasmus. The next year, Koch (2), in the well-known article describing the so-called Koch phenomenon, tested the reaction to subcutaneous injections of dead, as well as living tubercle bacilli, in normal and tuberculous guinea pigs. In normal animals he also produced local abscesses with dead organisms. However, in 1890, Wyssokowitsch (3) had also studied the effects of dead tubercle bacilli, using both subcutaneous and intraperitoneal injections in rats. He found nodules of epithelioid cells and giant cells and infiltrations with neutrophilic leucocytes; that is, the production of tubercles. These observations were repeated and extended by Prudden and Hodenpyl (1891) (4) and by Prudden (1891) (5), and were then repeatedly confirmed (6-11).

The fact that dead bacilli will produce the lesions of tuberculosis is of great significance and sets the disease apart from other infections. It demonstrates that, in the actual infection, dead bacilli, as well as living, may play a rôle and gives an especial interest to the study of the tissue response to chemical fractions isolated from the organism. Proteins, carbohydrates, and lipoids all play a part in these reactions, but the lipoids alone produce tubercles. These substances have the power to stimulate the formation of the epithelioid cell and its multinuclear form, the Langhans giant cell, which together are the essential structural units of the tubercle.

While chemical analyses of the organisms give only the vaguest clues

to the complex compounds through which tubercle bacilli disintegrate in the body, it is, nevertheless, remarkable how closely the cellular reactions to certain chemical fractions reproduce the tubercle and how many of the various accessory cellular responses can be elicited by other fractions.

In the reactions to the fractions of the earliest analyses, however, it is not possible to make a sharp discrimination between the effects of proteins, carbohydrates, and lipoids. The earliest analyses of tubercle bacilli showed a high content of phosphorus and of lipoids. Hammerschlag (12) isolated material soluble in alcohol and ether and found that it made up about 27 per cent of the weight of the organisms. This material, on analysis, yielded lecithin, fat, and a certain toxic substance. On injecting the material soluble in alcohol and ether, and thus containing lipoids, the animals died with the symptoms which have since been definitely related to proteins and carbohydrates. This indicates that his material was a mixture. Hammerschlag also isolated a protein from the bacterial residue. De Schweinitz and Dorset (13) found even more lipoidal material, namely, 37 per cent.

The earliest report of a study of cellular reactions to materials from tubercle bacilli is that of Weyl (14), in 1891, who extracted the bacilli with caustic soda and obtained material which caused necrosis. Auclair (15) and Auclair and Paris (16) made a series of interesting biological studies with fractions from the tubercle bacillus. They killed the organisms with sunlight. Auclair (1897) collected the volatile substances from the organisms, responsible for the characteristic odor of the cultures, and found them toxic. With small doses there was congestion and hemorrhage in the lungs and liver; in larger doses, death. In tuberculous animals there was a rise in temperature and a rapid loss of weight. In 1898 Auclair described the serious illness of eight people from the volatile products set free while the ether extract was being filtered. Koch (1897) (2) had already called attention to the danger of grinding tubercle bacilli in open mortars. The effects of these volatile products have been repeatedly experienced by those who have worked with this organism. In the experience of the H. K. Mulford Company which has handled enormous quantities of these organisms, the bovine organism liberates more of the volatile material than the other strains.

Auclair suspended the bacilli in ether and noted that two types of organisms could be demonstrated; cocci which floated in the ether and made it milky, and bacilli which sank to the bottom on standing. The

cocci, inoculated subcutaneously, gave small nodules which caseated. The bacilli, inoculated in the same way, gave lesions which were resorbed; abscesses were rare but the animals showed loss in weight. He found that ether extracted a lipid analogous to cholesterin; alcohol, a lecithin; and chloroform, a wax. The extracts obtained from ether and chloroform were both acid-fast as were the organisms after their removal. He found that all of the lipoidal substances gave cellular reactions comparable to those produced by the bacillus. Injected under the skin, the ether-soluble material gave abscesses which caseated. With intratracheal injections, it produced pneumonia with marked caseation. The chloroform-soluble material was slower in reaction; subcutaneously it caused sclerosis, and intratracheally a reaction like tuberculous pneumonia. From these results Auclair emphasized caseation as due to lipoids from the organisms. These biological reactions to the materials extracted by Auclair were then tested in different parts of the body (17-20). Dominici and Ostrovsky (21) extracted heat-killed tubercle bacilli with water and obtained substances which, injected subcutaneously, gave tubercular tissue in the organs. They concluded that lipoids were not essential for the production of tubercles, but inasmuch as the tuberculo-phosphatide separated by Anderson, to be described later, is soluble in water, it is possible that this aqueous extract contained not only protein and polysaccharide but also some phosphatide. With this aqueous extract they also produced a general stimulation of lymphoid tissues and showed a remarkable increase in lymphoblasts (see their plate XXXIII) in the lymph follicles.

The lipoidal material which Levene (22) isolated from the tubercle bacillus was tested at the Trudeau Sanatorium and produced sterile abscesses under the skin. Morse and Stott (23) and Ray and Shipman (24) isolated lipoids from tubercle bacilli for the purpose of studying the cellular reactions to them. Morse and Stott used first ether and then boiling alcohol for the extractions. Ray and Shipman retested these methods and used also the more effective methods of Long, namely, alcohol followed by hot toluol. These lipoids were then extracted by ether and by chloroform. With the lipoids extracted by these means, both groups obtained tubercular tissue consisting of epithelioid cells, giant cells, and fibroblasts, and recognized that the formation of the tubercle may be considered as a foreign body reaction toward tuberculo-lipoids. Although these are important steps, it is not possible to recognize the nature of the lipoidal material extracted and their reports of the biological testing are not conclusive because they used diluting

menstrua for the tuberculo-lipoids, olive oil in the one case and sodium stearate in the other, without adequate control of their reaction.

Acid-fastness in tubercle and paratubercle bacilli was from the very beginning a factor of great interest in connection with the chemistry of the lipoids of these organisms. That certain organisms possessed the property of resistance to decolorizing both with acids and with alkalies was first noted by Neisser; this property played an important rôle in the work of Koch, and was further analyzed by Ehrlich. Koch (2) (1897) noted that if tubercle bacilli were ground in a mortar they lost this property entirely. Benians (25) and Sherman (26) confirmed this and showed that acid-fastness could be made to disappear from tubercle bacilli by simply rubbing them between two slides. These observations suggest that if the property is due to a specific substance, a simple dispersion of the substance into sufficiently fine particles is sufficient to remove the reaction. As early as 1886, Bienstock (27) and Gottstein (28) both showed that the property of acid-fastness was associated with lipoids. Auclair found that all of the lipoidal substances, as he extracted them, were acid-fast; and that, after their removal, this property was still retained by the bacilli. It is now clear that Auclair's separation of the lipoids was partial and that the complete defatting of the organisms by lipoidal solvents is extremely difficult, if not impossible without the breaking up of the bacilli. Aronson (29) found that the wax from the organism is acid-fast; this was confirmed by Borrel (30) and by Bulloch and Macleod (31). Bulloch and Macleod showed that the substance which had this property was extremely resistant to saponification and was an alcohol. In 1910, Aronson showed that saponification of the entire organism with strong alcoholic caustic potash gave an unsaponifiable material which was acid-fast. He found also that it was a higher alcohol. This has now been shown in the more recent analyses of Tamura (32), Goris (33), and Anderson and his associates (34-42). In Anderson's analysis, it has proved that the material soluble in alcohol-ether entirely lacks the property of acid-fastness after the traces of unsaponifiable material have been removed from it.

Early in the study of tubercle bacilli (1883 and 1884), Malassez and Vignal (43) suggested that there is a non-acid-fast phase of Koch's organism. They found a non-acid-fast organism in a nodule from the skin of a child who had died of tuberculous meningitis, inoculated the material into guinea pigs and recovered acid-fast bacilli. Ferran (1897) (44) then showed that non-acid-fast forms could be developed in cultures of tubercle bacilli by reducing the amount of peptone and glycerin

in the media in each succeeding transplant. The cultures which he obtained in this way contained some acid-fast organisms and were of low virulence. In 1903, Auclair (15) repeated this experiment, but by omitting the glycerine altogether, he degraded a culture into forms which were wholly non-acid-fast and avirulent. Moreover, he was unable to cause these organisms to recover their virulence. He analyzed this culture by the same methods he had been using for the acid-fast forms, obtained the same type of acid-fast lipoids as from the virulent cultures, but in less amount. Moreover, these lipoids gave the same biological reactions as those from virulent tubercle bacilli. The occurrence of non-acid-fast forms of tubercle bacilli has now been repeatedly reported (45 to 57) since their first discovery by Malassez and Vignal and Ferran. Moreover, Kahn's observations (56, 57), in which he has isolated a single acid-fast bacillus from a culture of tubercle bacilli, watched it fragment into cocci and then still further subdivide into colonies of tiny, non-acid-fast organisms, and then regenerate the original form, make conclusive proof of a non-acid-fast cycle in the life of the tubercle bacillus. This fact enhances the interest in the early observation of Auclair that the non-acid-fast form contains an acid-fast lipid which can be demonstrated chemically.

In comparing the biological reactions to fractions from different chemical analyses, it is of the utmost importance to know the nature of the material used. In a recent extensive review, Albert-Weil (58) has made tables of many of the chemical analyses which elucidate the comparisons where possible (13-16, 22-24, 29-33, 59-68). He has presented charts which analyze the sequence of the procedures of Goris (38) and of Anderson (37-42) who have made the most extensive studies of the tuberculo-lipoids. Many factors make the comparisons of these analyses difficult: variations in the strains of the bacteria used, in the media on which they were cultivated, the age and the condition of the organisms when the analyses were made, the solvents used, and finally, as Albert-Weil has pointed out, the order in which they were applied. Perhaps the most important factor contributing to the difficulties in comparing different biological studies of the lipoids from different extractions has been brought out by Anderson, namely, that no one solvent makes a complete separation of one lipid from the others. For example, he found that a little of the unsaponifiable acid-fast material which is soluble in chloroform came down with the entirely non-acid-fast material soluble in alcohol and ether. This means that some of the materials of the earlier analyses were probably mixtures of the lipoids



separated by Anderson. No claim of purity, in the chemical sense, is made for the products of the recent analyses, but the products have been submitted to biological testing in succeeding stages of purification. In the case of some of these products, a relatively simple and entirely constant biological reaction has been observed.

Chemical analysis of the bacteria depended from the start on devising suitable media for the growth of the organisms. This was clear to Hammerschlag (12) who made the first analysis. He said that his analysis, made not long after the discovery of the organism (1882) (2) had to await the introduction of glycerin (Nocard and Roux, 69) into the media, which made possible an adequate growth of the bacilli. Hammerschlag used glycerinated bouillon and glycerin-peptone agar. In 1892, Kühne (70) showed that tubercle bacilli could be grown on simple media without protein, and in 1894 Proskauer and Beck (71) developed what is still regarded as the simplest formula on which growth is possible, namely, ammonium carbonate, mono-potassium phosphate, magnesium sulphate, and glycerol. Luxuriant growth is necessary for chemical analysis. Armand-Delille, Mayer, Schaeffer and Terroine (72) studied the nitrogenous compounds in the media and found that the monoamino acids were the indispensable part of the peptone, while in the bouillon certain extractives (carnosin, creatin, and sarcosin) and diamino acids (arganine and histidine) were important. Kendal, Day and Walker (73) then showed that in albumen-free media containing asparagin, glycerin, and 1 per cent of either mannite or glucose, the organisms could synthesize their nitrogenous compounds from the asparagin and their fats and waxes from the glycerin and sugar. The early work of De Schweinitz and Dorset (13) had already shown that phosphorus pentoxide made up 55 per cent of the mineral content of tubercle bacilli and Baudran (74) found that tubercle bacilli use the phosphates in the media. These studies were followed by more extensive research on the metabolism of the organism and the various substances necessary for their best growth (75-80). The facts disclosed by these investigations have led to all of the synthetic media, of which the Proskauer and Beck (71), the Sauton (81), and the Long (75, 76) are examples. Only by the use of such media can one be sure that all of the complex carbohydrates, lipoids, and proteins found in the bacilli and in the fluid media on which they have been grown have been synthesized by the organisms.

A recognition of the difficulties in comparing the results of previous analyses and of the fundamental importance of using standard strains

of organisms and synthetic media without protein led to a coöperative plan by the Research Committee of the National Tuberculosis Association, William Charles White, United States Public Health Service, Chairman. This plan was to select standard strains of organisms, have them grown in large quantities on synthetic media, analyzed chemically, and have the various products tested biologically to determine how many of the factors of the disease could be reproduced. Certain standard strains of tubercle bacilli, human, bovine, and avian, together with a strain of the timothy grass bacillus and a strain cultivated from a leprous lesion, were chosen. The strains used are shown in Anderson's tables (1932). The first work was done with the human strain H-37, and then fractionation by the same procedures was made of the others. The organisms were grown by the H. K. Mulford Company on Long's synthetic media made entirely from chemicals of the same standard lot, purchased at one time. All of the cultures have been grown in pyrex glass. No pains have been spared to make the cultural conditions as uniform as possible. Moreover, the organisms have been furnished to the chemists in quantities adequate for analysis. The analyses of the lipoids and carbohydrates related to them have been made by Dr. R. J. Anderson, with the aid of Drs. E. G. Roberts, E. Chargaff, M. L. Burt, M. C. Pangborn, and N. Uyei, Sterling Chemical Laboratory, Yale University. The proteins and carbohydrates of the bacilli have been extracted by Prof. T. B. Johnson, of Yale University, with the aid of Drs. R. D. Coghill, E. B. Brown, and A. G. Renfrew. Dr. M. Heidelberger, of Columbia University, has studied the several carbohydrates from the organisms. The bacillary proteins and carbohydrates found in the media have been isolated and tested biologically by Drs. E. R. Long and F. B. Seibert, of the University of Chicago. It has been our privilege to receive some of each of these fractions for biological study. A complete bibliography of this work up to 1929 has been published (82).

Proteins from tubercle bacilli are responsible for the reactions that give the skin test (83, 84). Injected intravenously they produce the changes in the cells of the circulating blood common to all other proteins, and give a rise of temperature in the normal animal. The tuberculous animal is remarkably sensitive to them; they give a rise in temperature or, after sufficient dosage, a rapid fall in temperature, followed by death. The cellular reaction to the proteins is mainly a proliferation of plasma cells (85). There is also damage to the endothelium of the vessels, causing hemorrhage (86). Injections of the tuberculo-polysaccharides may cause death in the tuberculous animal, and in the tissues of the

normal animal they are chemotactic and toxic to the neutrophilic leucocytes (86-89). The rise in temperature which follows the injection of polysaccharide is probably produced by an accompanying nitrogenous compound. The lipoids, on the other hand, produce a great variety of cellular reactions, reproducing many of the lesions characteristic of the disease (86-89).

CELLULAR REACTIONS TO TUBERCULO-LIPOIDS. With the abundant material furnished by the H. K. Mulford Company, Doctor Anderson (34-42) separated the lipoids of tubercle bacilli into a phosphatide, an acetone-soluble fat, and a wax. Each of these three substances gives rise to cellular reactions which are so constant and so characteristic that the material injected can be determined from the lesions.

I. *Cellular Reactions to Tuberculo-Phosphatide and Phthioic Acid. Phosphatide.* An alcohol and ether extract was first made from the bacilli. This material was then extracted with acetone, and a phosphatide which was insoluble in acetone and a fat which was soluble, were obtained. Traces of wax and fat were removed from the phosphatide and then it was further purified. The first preparation which we received from Doctor Anderson contained a few acid-fast bacilli in small clumps. After this lot, Doctor Anderson filtered the material through porcelain candles, a procedure which completely removed all of the acid-fast debris. The material is stable as far as can be judged from its biological reaction which has not changed in the material which was prepared in 1926. The phosphatide is a soft, white, granular substance. It has been examined for us under crossed Nicol prisms by Dr. R. W. G. Wyckoff of The Rockefeller Institute, who reported it to be predominantly crystalline. This, of course, does not mean chemical purity, which Doctor Anderson has not yet obtained. He found that the phosphatide from the human strain of tubercle bacilli, H-37, contained 0.36 per cent nitrogen, the state of which he has not yet determined. The phosphatides from the other acid-fast organisms contain different percentages of nitrogen, as shown in Anderson's table II (1932, 34). In 1920, Linossier (65) extracted phosphatides from tubercle bacilli which he said were sticky or crystallizable with difficulty. It may thus be concluded that the tuberculo-phosphatide is a material which can crystallize.

Another property of no less importance is that when distilled water is first applied to the phosphatide, it breaks into typical myelin figures, looking much like the material in the sheaths of fresh, medullated nerve fibers, except that it is less refractive. This property is of great interest in the biological tests, because it enables one to identify the material

after it has been phagocytized by cells. The phagocytic cells of the connective tissues have been extensively studied both in their reactions toward other damaged cells, such as leucocytes in the tissues and free red blood cells, and toward insoluble particles, such as carbon, trypan blue, and carmine. These particles, which the cells have no power to disintegrate, have been used experimentally because they can be identified. To such experimental materials may now be added the tuberculo-phosphatide, which can be identified by the myelin figures, if examined soon after it has been phagocytized. Moreover, one may follow to some extent the signs of the breaking up of this material within the cells, and so study the processes by which the epithelioid cell of tuberculous infections is produced.

In the fresh state, this myelin-like material stains faintly pink in neutral red; after it has been taken into cells, this characteristic stain is retained for a time. In rabbit tissues the phagocytic cells soon secrete a fluid around the myelin-like masses making them stain red in this dye and obscuring the lipoidal material. In the guinea pig, on the other hand, the cells can be found engorged and distended with entirely unchanged myelin-like masses for at least twenty-four hours after the injection of the material. The description of the processes through which the cells which have phagocytized masses of phosphatide become typical epithelioid cells, will follow. These observations, based on the identification of the phosphatide within a phagocytic cell, establish the point that the epithelioid cell represents a foreign body reaction; thus, the epithelioid cell is the final stage of a cell which has taken in, and in part disintegrated, a foreign lipid.

The next step in these biological studies was to determine which type of cell of the connective tissues phagocytizes this material and thus gives rise to epithelioid cells. For this purpose the omentum offers great advantages. It can be separated into its two layers and spread as a film on a slide, allowing the study of the cellular changes in the living state without any distortion of the position of the cells. In no other place in the body are the cells of the connective tissues to be seen in simpler form and arrangement. In the normal omentum there are small masses of cells closely packed together, forming the so-called milk spots. The milk spots are composed of monocytes and of young connective tissue cells, somewhat less differentiated. They have basophilic cytoplasm and contain a few vacuoles and many mitochondria. There may also be the simplest type of connective tissue cell, the so-called reticular cell, in which no differentiation of granules or vacuoles

in the cytoplasm can be detected. At times there are some lymphocytes and an occasional branched clasmatocyte. Similar small collections of young connective tissue cells, true milk spots, are to be found everywhere in the connective tissues, as was shown by Möllendorff and Möllendorff (90). In the interspaces between the milk spots of the omentum are scattered branched cells of the macrophage or clasmatocyte type. They always contain some debris in their vacuoles, for they are constantly functioning with reference to substances which are passing through the omentum. These clasmatocytes of the interspaces are the cells which engorge themselves with any of the particulate materials used experimentally for the identification of these cells. They also engulf in large numbers any dead leucocytes and red cells that get free into the tissues. The mass of material which these cells will take up is important in determining their functional nature as macrophages or "big eaters," since a monocyte or even such cells as the secretory cells of the liver will take in a small amount of any of the substances with which the clasmatocyte will engorge itself.

The cells of the milk spots have more deeply basophilic cytoplasm than the clasmatocytes around them and they also have massive amounts of mitochondria, signs which are recognized as characteristic of young cells in the connective tissues. The experiments with the tuberculo-phosphatide show that it is these young cells of the milk spots which phagocytize this material in massive amounts rather than the mature clasmatocytes adjacent to them.

The tuberculo-phosphatide, injected into the peritoneal cavity as a suspension in distilled water, is taken in by the clasmatocytes, as seen in the omentum, only in small amounts, so that there is only a slight change in their activity and no change in their shape; but it is phagocytized in large amounts by the cells of the milk spots. Lymphocytes, if present, are not involved. Within three days after the injection, the young connective tissue cells and monocytes become greatly increased in size and to some extent in numbers, so that the milk spots appear swollen to the unaided eye. When these cells are stained with neutral red, it appears that the lipoidal material has been segregated within a few vacuoles in the cytoplasm. Some of these cells with highly vacuolated cytoplasm wander into the peritoneal fluid, showing that they are motile; they may also divide. In this state these cells look like clasmatocytes, but it can be shown that they were derived from the monocytes of the milk spots and not from cells which were functioning as clasmatocytes before the experiment began. They represent a stage in the transformation of monocyte into epithelioid cells.

The cells which have engorged the material appear to break it into finer and finer particles and these gradual processes we have arbitrarily described in three stages. The first stage has the largest vacuoles filled with the phosphatide, either intact or slightly changed. These vacuoles are of irregular size and shape. For convenience we shall call this stage the "phosphatide cell." In the rabbit treated with the phosphatide from the human organism H-37, this first stage lasts three or four days.

The second stage shows the cytoplasm filled with a rosette of many coarse vacuoles, uniform in size, making the fixed cell appear to have a foamy cytoplasm. We speak of this stage as the coarse-vacuolated epithelioid cell; it is reached in five to seven days. The typical epithelioid cell is the third stage and has a rosette of such tiny vacuoles that the cytoplasm in the fixed cell appears dense and nearly uniform in structure. This stage is reached in the second week. The rabbit cells break down the phosphatide from H-37 in a uniform manner, the vacuoles being of approximately the same size in each cell at any given time. In this regard there are marked variations with the phosphatides from the other acid-fast strains.

These different stages in the development of epithelioid cells after the injection of tuberculo-phosphatide have been illustrated in part by Sabin, Doan, and Forkner (89). They will be shown further in a forthcoming paper by Smithburn and Sabin (91) from a study of the reactions of cells to the phosphatides from the various strains of acid-fast bacteria.

With repeated intraperitoneal injections of the phosphatide there is a marked maturation of monocytes and the production of epithelioid cells in such numbers that the outlines of the milk spots become obscured. That the phosphatide from the human strain is not greatly irritating to the cells of the rabbit is shown by the fact that leucocytes are called from the vessels only after the first injection; emigration of leucocytes does not occur after succeeding injections, and those present after the first injection are quickly phagocytized by clasmatocytes and destroyed. There is an increase in undifferentiated connective tissue cells in the omentum with repeated injections of the phosphatide, but it is minimal in the rabbit. In the guinea pig this reaction is much more marked. After from ten to fourteen daily doses of the phosphatide, epithelioid cells are to be found in large masses, in circumscribed tubercles, and in tubercular granulation tissue. Injection of phosphatide into the pleura or directly into the lung in the rabbit brings out with

especial clearness the fact that the epithelioid cell is the characteristic response to this material.

The response to the tuberculo-phosphatide is clearly the phagocytosis of this material. Other immediate reactions are minimal and consist only in a transient emigration of leucocytes from the tissues. However, important cellular reactions always develop during the second week after the introduction of this material into the tissues; the occurrence of large numbers of Langhans giant cells, the local stimulation of lymphocytes and of plasma cells, and the process of caseation.

The Langhans giant cell, as seen in supravital reaction, has the same type of rosette as the typical epithelioid cell of the third stage. Forkner (92—see also 89) has shown that this type of giant cell is a multinuclear epithelioid type, while the so-called foreign body type is produced in the reaction to foreign material by the fusion of cells, usually monocytes. In the early reaction to the phosphatide, there is a marked tendency for epithelioid cells with two nuclei to occur and by the second week there may be great numbers of Langhans giant cells; in certain animals some of the lesions of the omentum may be predominantly of epithelioid giant cells and frequently the reaction in the retrosternal lymph nodes is made up entirely of these types. The epithelioid giant cell never shows the first stage of the phagocytosis of the lipid and almost never the stage of the coarse vacuoles. It is a typical epithelioid cell which has become multinucleated. These giant cells show no especial relationship to caseation.

In every experiment in which as much as two weeks have elapsed after the injection of the phosphatide, the tissues have been found with considerable infiltrations of lymphocytes. In some cases the tubercles have had complete capsules of lymphocytes like those to be seen in the actual infection. The areas in which giant cells predominate are likely to show many lymphocytes as well. At present we are unable to analyze this late association of lymphocytes with epithelioid cells in the reaction to the phosphatide, but we have seen no evidence that it is an immediate and direct effect of the phosphatide. In this connection the observations of Dominici and Ostrovsky must be recalled (21).

The epithelioid cell may remain for long periods of time; some have been found six and one-half months after the injection of the lipid, but in every experiment with any of the phosphatides from the acid-fast bacilli, we have found considerable amounts of caseation during the second week. We have not designated any nodule caseous unless it showed clearly a border of intact epithelioid cells around a center of

necrotic tissue; indeed, in many of the caseous nodules the outlines of the dead epithelioid cells are still present in the center, surrounded by leucocytes. It is our view that caseation is the end stage of the epithelioid cell, the infiltration of the leucocytes being secondary to the death of these cells. Thus the so-called "caseation fraction" of Auclair would be interpreted by us as a material which had produced epithelioid cells. Whether or not the death of epithelioid cells is hastened by tuberculo-protein is being investigated. Caseation is more extensive in the tissues of the guinea pig than in those of the rabbit, after the phosphatide from H-37.

In general the reactions to the phosphatide from the bovine and avian strains of tubercle bacilli, and the timothy grass bacillus and the lepra strain are like, in kind, to those from the human tubercle bacillus but differ in the time which the cells require to break down the lipid. The reaction to the avian phosphatide is most like that to the human. It differs in that the intracellular dispersion of the material is a little slower, so that at the end of ten days, the epithelioid cells, which are present in massive numbers, are largely in the second stage rather than in the third, that is to say, they are epithelioid cells with coarse vacuoles. There may even be a few cells still in the first stage. There may be only a few giant cells, or, in other animals, they may occur in massive numbers. There is a marked tendency for these giant cells to be of complex type. Instead of being typical epithelioid giant cells of moderate size and with peripheral arrangement of nuclei, these cells are large, have one or more wide cytoplasmic areas corresponding to the rosette of the epithelioid cell, and great masses of nuclei closely packed together either in the center or at one end. They may have resulted from the fusion of several Langhans giant cells. In the reaction to this phosphatide there are quite extensive masses of plasma cells and the same infiltration with lymphocytes as with the material from the human tubercle bacillus. Caseation is marked in the areas of epithelioid cells, whether in the subcutaneous nodules or in the omentum, as well as in the lymph nodes draining these areas.

The reactions of the cells of the rabbit toward the phosphatide from the bovine organism and from the timothy grass bacillus are much alike; these materials are broken down more slowly than the phosphatide from H-37 and from the avian bacillus. In the lesions which have been produced by the bovine phosphatide, there are marked variations in the epithelioid cells, even after two weeks; in some areas these cells are of the third stage with enormous numbers of giant cells; in other areas



the epithelioid cells are in all three stages, showing an irregular breaking down of the phosphatide. There may be an increase in undifferentiated connective tissue cells, and more infiltration with leucocytes, lymphocytes, and plasma cells than after the phosphatide from H-37.

Two weeks after the injection of the phosphatide from the timothy grass bacillus there seems to be less formation of epithelioid cells but this is only because all of the large nodules have become caseous; the diffuse reaction of intact epithelioid cells shows the same mixed types as after the bovine phosphatide. After ten doses of the former, many of the epithelioid cells are still in the second stage and some even in the first. The material seems to be more irritating than any of the other phosphatides, for even after one intraperitoneal injection there are several abscesses in the omentum; and in the later reaction there are many leucocytes scattered in the tissues and phagocytized by clasmatocytes. There are also many plasma cells.

The study of the phosphatide from the lepra organism is incomplete since only small amounts have been available. The material is more irritating and the tissues show more of an increase in connective tissue cells. Also there has been much more variation in the reaction of different animals. In one experiment there was a considerable diffuse reaction of very complex epithelioid cells, a few of them being in small clumps or tubercles. In the regional lymph nodes there was, however, a massive reaction of epithelioid giant cells, a few of them being the compound types. There was also some caseation and a general infiltration of the tissues with leucocytes and many clumps of plasma cells. In another animal the reaction was almost wholly of leucocytes, enormous numbers of them having been called from the vessels and phagocytized by clasmatocytes.

From this study with the phosphatides of the acid-fast organisms, we have designated the reaction of the material toward the production of epithelioid cells and their multinuclear form, the Langhans giant cells, as the specific reaction toward tuberculo-lipoid. By this use of the term "specific reaction" is meant to imply that emphasis should be put on the epithelioid cell, either in mono- or multinucleated form, as the one structure which is essential in tubercles or in tubercular infiltrations or in tubercular granulation tissue. The phosphatides from all of the acid-fast organisms included in this study produce epithelioid cells and Langhans giant cells which go on to caseation. Caseation is, therefore, a part of the specific reaction.

All other reactions, such as the calling of leucocytes from the blood

vessels, their phagocytosis and destruction by clasmatocytes, the development of plasma cells, the increase in lymphocytes, in young connective tissue cells, in fibroblasts, and the development of new blood vessels, we have termed non-specific. On this basis, it is clear that the phosphatide from the human strain of tubercle bacilli gives the more specific response, while all of the other phosphatides give more non-specific reaction as well, the reactions to the phosphatide from the timothy grass bacillus and the lepra strain being the more mixed.

Of the various controls only one material so far tested acts just like the tuberculo-phosphatide, namely, lecithin. A quantity of this substance was prepared for us from the brain, by Dr. P. A. Levene. The lecithin is phagocytized by the same mononuclear cells as the tuberculo-phosphatide and produces epithelioid cells and giant cells in massive tubercles. In the original report (89) it was stated that only a small reaction was found in the omentum. Subsequent studies of sections from many places in the peritoneal cavity have shown an extensive reaction elsewhere. A dilauryl acetic acid containing the same number of carbon atoms as the phthioic acid, namely 26, synthesized for us by Prof. Roger Adams, Department of Chemistry, University of Illinois, is also phagocytized by the cells in the milk spots rather than by clasmatocytes, but this material does not change these cells into epithelioid types in the same period of time.

An important property of the tuberculo-phosphatide is that it acts as an antigen. This reaction was first described for alcoholic extracts of tubercle bacilli by Meyer (93); it has been studied for methyl extracts by Boquet and Nègre (94). Pinner (95) showed the phosphatide by Anderson to be antigenic, and further studies of this reaction have been made by Doan (96), and Doan and Moore (97).

*Phthioic acid.* All of the primary divisions of the lipoids, the phosphatide, the waxes, and the acetone-soluble material yielded on analysis certain hitherto unknown, saturated fatty acids of high molecular weight. A fatty acid designated Fatty Acid I, from the phosphatide A-3, of the human strain of organisms, was tested first in the rabbit. All of the tendency of the phosphatide toward the production of epithelioid cells was carried over into this acid; and this acid produced as great an amount of epithelioid cells as the original phosphatide (see table 1, in 88). Tubercles surrounded by lymphocytes were characteristic of the reaction. It was, however, more irritating than the phosphatide, there was a greater increase in connective tissue cells, and there were large abscesses. Typical caseation, in the sense we have defined

it, was present only in small foci. The cellular reactions simulated those of the disease more closely than those after the phosphatide. On further analysis of Fatty Acid I, Doctor Anderson obtained a hitherto unknown fatty acid of high molecular weight, which has been found in the biological test to be the only substance from the original acid producing a response of tubercular tissue. Doctor Anderson therefore named it phthioic acid. The empirical formula of this acid is  $C_{26}H_{52}O_2$ . The phosphatide also yielded glycerophosphoric, oleic, and palmitic acids. Each of these was tested by intraperitoneal injections and gave leucocytes both free and in clasmatoocytes, some increase in blood vessels, and a non-specific thickening of the omentum in varying degrees.

The phthioic acid was a heavy oil and proved much too irritating to be used undiluted; it caused necrosis and gave very complex cellular responses. In spite of this complexity of these reactions, it could be made out that there was a marked maturation of monocytes. It was necessary, therefore, to find a bland oil for a diluting menstruum; olive oil was tried and rejected on account of its extreme irritation of the connective tissues. Mineral oil was chosen because, though not inert, it gave only a moderate increase in fibroblasts and a minimal production of epithelioid cells. Diluted in mineral oil, the phthioic acid from the phosphatide A-3 gave a diffuse reaction of epithelioids as well as typical small tubercles, like those from the phosphatide and closely simulating those of the disease. None of the highly vacuolated types of cells produced by the phagocytosis of the complex lipoid appeared with the fatty acid; the epithelioid cells were all of the typical third stage. These results may be interpreted as indicating that the original phosphatide in these experiments was phagocytized by cells and reduced to some state comparable to the fatty acid. In the one case the degradation of the material was done within the cell and in the other in the test tube. However, it is probable that in the disintegration of the bacilli that occurs in the actual infection, the phagocytosis of the material by cells takes place when the substances are in the more complex molecule. Interesting evidence bearing on this point is to be found in the work of Bickford (98) who has shown that there is a specific type of early epithelioid cell in the meninges which is produced by the injection of living or dead tubercle bacilli or tuberculo-phosphatide.

The fatty acids from the purified and soft waxes also produced typical tubercles in varying amounts, but not as massive reactions as that from the fatty acid from the phosphatides. The acetone-soluble material contained more of these fatty acids than the other lipoids. Doctor

Anderson separated these fatty acids and found an isomer of stearic acid, which he called tuberculo-stearic acid, and phthioic acid. The former was irritating but did not produce tubercular tissue.

The acetone-soluble material yielded enough phthioic acid for fractionation and Doctor Anderson separated the acid from this source into a dextro- and a levorotatory form. The specific activity toward the production of epithelioid cells was carried only in the dextrorotatory acid. The response to both of these acids is complex and the actual increase in new tissue is fully as great with the levorotatory acid. Introduced intraperitoneally in mineral oil, the levorotatory form gives a marked increase in thickness of the omentum and subperitoneal tissues. There is an increase in young connective tissue cells, an infiltration of the tissues with leucocytes, much phagocytosis of them by clasmatocytes, an increase in lymphocytes and plasma cells, together with the development of many monocytes but practically no epithelioid cells. After the dextrorotatory acid, on the other hand, there is also a marked production of epithelioid cells, and a few epithelioid giant cells; there is relatively little tendency toward the production of these cells in clumps or tubercles and we have not found these cells surrounded with lymphocytes. There is the same complex non-specific reaction toward leucocytes and plasma cells with the dextrorotatory acid as with the levorotatory, but the presence of the epithelioids in the one case makes such a difference in the appearance of the tissues that the type of acid used can be determined from the tissues without reference to records.

The question has been raised by Boissevain and Ryder (99) as to whether the alcohol-ether soluble material isolated by Anderson is really a phosphatide or merely debris of bacilli, and therefore whether the biological reactions which we have recorded with this material are necessarily associated with lipoids. The fact that dead tubercle bacilli also produce tubercles makes this question pertinent. Acid-fast debris has not been present in any of the phosphatides prepared by Anderson after the first lot; the filtering through candles removed all demonstrable acid-fast masses. Therefore the question at issue is the presence of non-acid-fast debris. It is possible that this question cannot be wholly settled until the nature of the nitrogen present in the phosphatide has been determined and until complete purification of the material has been accomplished, but the evidence up to the present time in favor of the hypothesis that the epithelioid cell is a foreign body reaction to tuberculo-lipoid is as follows. Epithelioid cells have been produced only by extracts which have been obtained by lipoidal solvents, not by

tuberculo-proteins or polysaccharides; thus if the epithelioid cell can be produced chemically at all, it must be by lipoids. The predominantly crystalline nature of the dry phosphatide and its transformation immediately on wetting into myelin figures are against the view that this material is amorphous debris. The identification of the phagocytosis of this myelin-like material by cells in a definite location, namely, in the milk spots of the omentum, so that the reaction can be followed into typical epithelioid cells, gives evidence that the epithelioid cell represents a foreign body reaction toward a lipid. The production of the epithelioid cell in the same location by pure lecithin also suggests that the epithelioid type may be a form of reaction toward ingested lipoids.

Doctor Boissevain has also brought up the interesting question of the discrepancy between the amount of epithelioid tissue produced by dead tubercle bacilli and the corresponding amount of phosphatide on the basis of its being 6 per cent by weight of the organisms. These two quantitative reactions cannot be compared for the phosphatide contains only a part of the specifically active lipid. Doctor Anderson has shown that all of the lipoids extracted by solvents, the alcohol-soluble, chloroform-soluble, and the acetone-soluble material, contain phthioic acid; moreover, lipoidal solvents do not remove all of these substances, for the so-called "defatted bacilli" are still acid-fast and produce epithelioid cells, in less amount, however, than intact dead bacilli. The total lipid can only be extracted by methods which completely disintegrate the bacilli. If the total lipoidal material were available, comparative tests between the amount of the epithelioid reaction to this material and dead bacilli could be made. For such tests, however, the intraperitoneal route is not as good as the subcutaneous on account of the wide dispersion of the reaction in the peritoneal cavity. In this area the reaction is produced not only in the omentum but under the serosal lining of the body-wall, the wall of the intestine, in the mesentery, the diaphragm, in the capsules of the liver and spleen, and around the reproductive organs. The cellular reactions are also reflected by the cells free in the peritoneal fluid and always appear in the retrosternal lymph nodes which drain the peritoneal cavity. They are not found in the mesenteric lymph nodes unless the bowel wall has been involved. The value of the intraperitoneal route is the opportunity to follow the cellular reactions in living tissue without any distortion in the arrangement of the cells. In subcutaneous tests, however, all of the cellular reactions are in a restricted area except that which is to be found in the regional lymph nodes.

There are, however, certain quantitative discrepancies in our results which cannot be explained at present. In all of the tests with the more purified forms of the phthioic acid there has been a marked loss in the amount of specific activity from the reaction of the corresponding amount of the original phosphatide and the mixture of fatty acids extracted from the phosphatide. Moreover, the acetone-soluble material which contained more of the phthioic acid than the phosphatide itself shows little tendency for the epithelioid cells to be in large masses or tubercles. The question as to whether these phenomena are due to any accessory factors cannot be answered at the present time.

II. *Cellular Reactions to the Unsaponifiable Wax.* Doctor Anderson considers the so-called waxes to be very complex phosphatides. From them he isolated an unsaponifiable material having the property of acid-fastness which discriminates the group of the tubercle and paratubercle bacilli. Doctor Anderson has found this material to be a higher alcohol with the formula of  $C_{94}H_{188}O_4$  and has discussed the relationship of this material to the higher alcohols of other analyses. We have had the privilege of studying also a similar acid-fast material prepared from tubercle bacilli by Dr. P. A. Levene. Both specimens were completely insoluble in water and had therefore to be suspended in mineral oil. In this menstruum they produced identical reactions, namely, a marked production of young connective tissue cells, both diffusely and in small clumps. There has been no sign that this material is phagocytized by any of the cells. The new cells which appear after the injection of this material are round or oval and of about the size of monocytes. Little differentiation can be made out in the cytoplasm which is slightly basophilic. However, the cytoplasm is so delicate that it cannot be made out at all in sections, so that the clumps or pseudo-tubercles of them look like masses of large nuclei without cytoplasmic outlines around them. There are considerable numbers of leucocytes scattered diffusely and infiltrating the pseudo-tubercles. Similar clumps of cells characterize the reaction to the levorotatory phthioic acid. Thus the cellular reaction to the unsaponifiable alcohol is a double one: undifferentiated connective tissue cells and leucocytes.

III. *Cellular Reactions to the Acetone-Soluble Fat.* By far the most complex cellular reactions are produced by the acetone-soluble fat. We have tested this material from the human, bovine, and avian tubercle bacilli and from the timothy grass bacillus. The supravital studies show that every type of connective tissue cell has been stimulated. The analysis of this material by Doctor Anderson shows that it is a complex

mixture of fatty acids, butyric, palmitic, stearic, cerotic, linoleic, linolenic, tuberculo-stearic, and phthioic acids. Corresponding to the phthioic acid there is a diffuse reaction of epithelioid cells; besides this, there is a general infiltration of the tissues with leucocytes, and many of them are in clasmatocytes; there is also a great increase in undifferentiated connective tissue cells, fibroblasts, lymphocytes, plasma cells, and a marked increase in new blood vessels with hemorrhage. The diffuse character of the epithelioid reaction may be associated with the intensity of these non-specific reactions. Further studies with the material from the various acid-fast strains of organisms are necessary in order to analyze the complexity of these reactions, which may be due in part to the high acidity of the material. As a control for this part of the lipoid, we have tested the acetone-soluble lipoid prepared from the streptococcus by Doctor Heidelberg. It appears to be the only type of lipoid in this organism; it is present only in small amounts in these organisms but gives very complex cellular responses with, however, no epithelioid cells.

From these studies of cellular reactions toward tuberculo-lipoids, the mass of evidence seems to indicate that they are remarkable stimulants of the cells of the connective tissues. Some of this material, such as the phosphatide, seems to act as a foreign body and produces effects through being phagocytized by cells; other materials, such as the unsaponifiable higher alcohol, act as a stimulant without being phagocytized; and the third lipoid, the acetone-soluble fat, is an extremely complex irritant. Reason for the cellular lesions in tuberculosis can be found in all these reactions. The view that the epithelioid cell is the result of the phagocytosis of a tuberculo-lipoid by monocytes and young connective tissue cells is in agreement with the view that the tubercle in the disease arises locally from fixed connective tissue cells.

CELLULAR REACTIONS TO POLYSACCHARIDES. Polysaccharides obtained from the lipoids by Doctor Anderson and from the whole bacillus by Doctor Heidelberg have been tested on the cells of the connective tissues. All of these materials give the same effect, namely, the calling of leucocytes from vessels and the damaging of them so that they are quickly phagocytized by clasmatocytes. This is a constant reaction and occurs regardless of the number of injections. There is no substance tested from the tubercle bacillus that did not call leucocytes from the vessels and in some instances this has been an extreme reaction in response to some of the lipoidal fractions, as for example, the acetone-soluble fat. However, this reaction is so consistently found with all

of the polysaccharides as to raise the issue as to whether the material in the other fractions which so damages the leucocytes that they are quickly taken in by clasmatocytes may not be the content of carbohydrate.

**CELLULAR REACTIONS TO PROTEIN.** It was shown by Doctor Miller (85) that the various preparations of protein obtained from the tubercle bacilli all have a remarkable power toward the production of plasma cells. With repeated injections of the protein he could follow the complete life cycle of these cells, producing its youngest stages, the well-known mature, Marschalkow type, and the final stage, the so-called Russell body cell. Of all of the materials from the tubercle bacillus, the tuberculo-protein gives plasma cells in the most massive amounts. They are found, however, in considerable numbers after repeated injections of the polysaccharide; and, as has been stated, they are increased over normal numbers after the phosphatide, especially from the organisms other than the human. It is thus interesting to speculate as to whether a response of plasma cells to polysaccharide or lipid may be a biological test of the presence of some nitrogenous compound.

#### SUMMARY

In these studies on the cellular reactions to chemical fractions from the tubercle bacillus, it has been shown that there are three different types of complex lipoids in the organism which can be discriminated by the cellular reactions they produce.

The phosphatide reproduces the tubercle; it is phagocytized by certain cells of the connective tissues, namely, by monocytes, and partially degraded within them, thereby forming the epithelioid cell. The fact that this material is phagocytized, and good evidence of this is obtained by seeing the characteristic myelin figures of the original material within the living cell, goes far to indicate that it is the substance itself and not some contaminating impurity which is responsible for this action.

The only constituent of this phosphatide which can produce this reaction is a saturated fatty acid of high molecular weight, phthioic acid, of the formula  $C_{26}H_{52}O_2$ . All of the other lipoids of the original fractionation, the wax and the acetone-soluble fat, contain also some of the phthioic acid, and therefore possess varying degrees of specific biological activity.

The waxes contain phthioic acid and an unsaponifiable residue which



is a higher alcohol,  $C_{94}H_{188}O_4$ . This unsaponifiable base of the wax does not seem to be phagocytized by cells, but in spite of its insolubility in water, when injected in an oil, acts as a remarkable stimulant toward the production of undifferentiated connective tissue cells. It is always irritating and calls leucocytes from the vessels. These two exceedingly complex phosphatides, the tuberculo-phosphatide and the waxes, then, may be considered as the types of lipoidal substances especially characteristic of the acid-fast strains of the organism, the phosphatide and the phthioic acid being responsible for the epithelioid cell—so prominent a factor in the cellular reactions of the disease, while the unsaponifiable material is responsible for the acid-fastness of the bacillus.

The especial interest of the acetone-soluble material is that it may be more like the lipoids of other strains of organisms; the extremely varied cellular reactions which it produces may be due to the fact that it is a complex mixture of fatty acids.

The characteristic cellular response to tuberculo-protein is the plasma cell. The tuberculo-polysaccharides are chemotactic and toxic to neutrophilic leucocytes.

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## THE EFFECT OF ANTITESTICULAR SERUM ON THE ENHANCEMENT VALUE OF TESTICLE EXTRACT

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A factor can be extracted from testicle tissue and sperm which is very active in enhancing infections (1-3) on the one hand, and in inhibiting the growth of transplantable tumors (4) on the other. The purpose of the present work has been to study the effects on this factor of sera from animals experimentally immunized to testicle extract, in order to gain further information as to the nature of the latter.

### *Material and Methods*

The testicle extract was prepared by grinding the glandular tissue with sand in an equal volume of Ringer's solution. After centrifugation the supernatant fluid was used.

Throughout the experiments the neuro strain of vaccine virus was employed as the infective agent. A rabbit was inoculated in the testicle and 5 days later the testicles were removed and ground with 50 cc. of a mixture in equal parts of glycerine and Ringer's solution. This suspension was diluted 50 times just prior to inoculation.

Antisera for the extracts of rat and rabbit testicles were prepared as follows: Rabbits were given 12 intravenous injections of an extract of normal testicles in increasing amounts, beginning with 0.2 cc. and ending with 1.2 cc., the same amount being given twice, with an interval of approximately 3 days between the injections. 10 days after the last one, the rabbits were bled for serum.

### *Effect of the Antiserum for Rabbit Testicle Extract on Vaccine Virus Infection*

*Experiment.*—3 rabbits, 3-15, 3-16 and 3-20, were injected with rabbit testicle extract and bled as described above. 0.5 cc. of each serum was mixed with 0.25 cc. of the diluted virus suspension, incubated for 2 hours at 37°C., and injected intradermally in rabbits. As controls, normal sera from Rabbits 3-15, 3-16 and 3-20, taken before immunization, were mixed with the virus, incubated and injected intradermally in the test animals, as was also an equivalent amount of vaccine virus alone. The relative extent of the lesions produced is shown in Table I.

In addition the three sera were tested with testicle extract for precipitins by the usual methods. No reactions were noted in dilutions higher than 1:1, and even at this dilution the results were questionable.

There was no difference of note between the extent of the lesions produced by the virus alone and by virus with normal serum, but those resulting from the virus with antitesticle serum were definitely smaller. The result might suggest that the serum had antivaccinal properties, but the fact that the virus used had been cultured in the rabbit testicle and that the serum had been procured by immunization with extract of rabbit testicle complicated the interpretation of the results.

TABLE I

*Effect of Antiserum for Rabbit Testicle Extract on Infection with Vaccine Virus*

Serum No.	Virus + serum taken before immunization	Virus + serum taken at middle of immunization	Virus + serum taken after immunization	Lesion from virus alone
3-15	+++	+	+	+++
3-16	++	+	+	+++
3-20	+++	+	+	+++

The number of pluses indicates the extent of the lesion.

#### *The Effect of Anti-Rat Testicle Serum on Vaccine Virus Infection*

In order to eliminate the complicating factor just mentioned, the test has been repeated, substituting rat for rabbit testicle extract.

*Experiment.*—3 rabbits, 3-18, 3-19 and 3-22, were injected with rat testicle extract and the serum collected, using the methods described above. In addition, a fourth rabbit, No. 8-3, was injected intraperitoneally with increasing doses of the same extract until it had received 31 cc., and it was then bled for serum. The presence of precipitins was tested with increasing dilutions of the sera against rat testicle extract at a constant dilution of 1:10. The mixtures incubated for 2 hours at 37°C. were read after standing overnight in the ice box. The results are shown in Table II. The serum from Rabbit 8-3, which had received the largest amount of antigen, gave the highest titer. The specificity of the reaction was shown by additional tests with the serum against rabbit testicle extract, which resulted in no precipitation.

The first three sera were next tested for their influence on vaccine virus infection. 0.5 cc. of each was mixed with 0.25 cc. of the diluted vaccine virus suspen-

sion, incubated for 2 hours and injected intradermally in rabbits. As controls each rabbit received in addition an injection of the virus alone and the virus with normal serum. The results of this experiment with the relative size of the lesions produced are shown in Table III.

As a further test of the influence on the virus infection of the antiserum for rat testicle extract, the serum with the greatest precipitating power, No. 8-3, was injected intradermally and 1 to 2 hours later vaccine virus was inoculated into the

TABLE II

*Precipitating Power of Antiserum for Rat Testicle Extract against the Homologous Antigen*

Dilution of serum.....	1:1	1:10	1:20	1:40	1:100	1:200	Antigen alone
Serum No.							
3-18	+++	++	+	+	±		
3-19	+++	+	—	—	—		
3-22	+++	++	+	±	—		
8-3	++++	+++	+++	++	++	±	—
Normal serum	—	—	—	—	—	—	—

The number of pluses indicates the degree of precipitation.

TABLE III

*Effect of Antiserum for Rat Testicle Extract on Infection with Vaccine Virus*

Serum No.	Virus + serum taken before immunization	Virus + serum taken at middle of immunization	Virus + serum taken after immunization	Lesion from virus alone
3-18	+++	+	+++	+++
3-19	++++	++++	++++	
3-22	++	+++	+++	

The number of pluses indicates the extent of the lesion.

same area. The test was controlled by the injection of normal serum, followed by virus inoculation. Also the virus and Serum 8-3 were injected immediately after mixing, thus eliminating the incubation period of the foregoing experiment. In none of these tests could any influence be noted on the size of the lesions produced by the virus.

It is evident from this group of experiments that antiserum for rat testicle extract has no effect on vaccine virus infection in the rabbit. This result seems to indicate that the inhibiting action noted in the



previous experiment was due to an interaction between the antiserum for rabbit testicle extract and some compounds of the testicle material containing the virus, not to a direct influence of the serum antibody on the virus.

*Effect of Antitesticular Serum on the Enhancing Power of Testicle Extract*

The factor in testicle extract which is responsible for the enhancement of infections is non-specific in that extracts of tissues from animals of other kind than that infected are just as effective as extracts from the same species. The action of the antisera on this enhancement factor was next investigated.

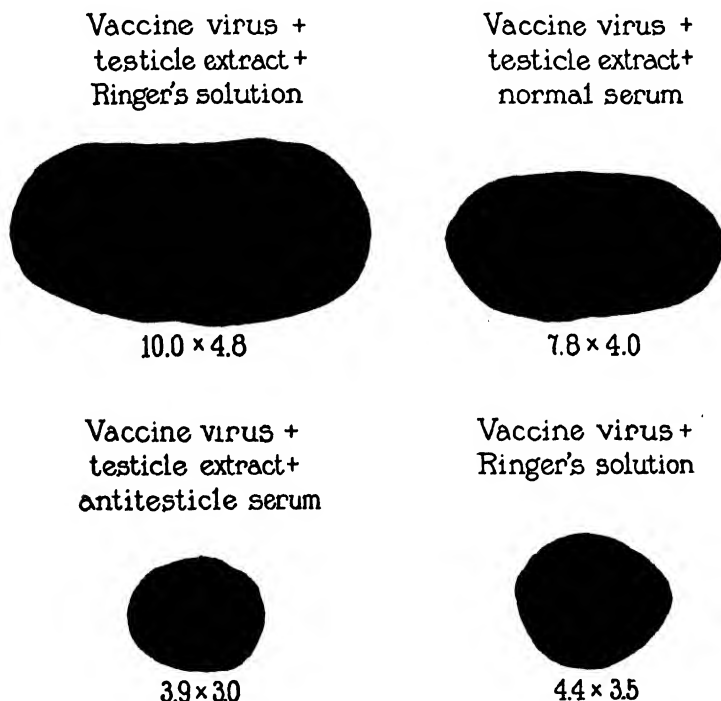
*Experiment.*—To 5 tubes, each containing 5 cc. of rat testicle extract diluted 1:10, were added respectively 1 cc. each of anti-rat testicle extract Sera 3-18, 3-22, 8-3, normal rabbit serum and Ringer's solution. The mixtures were shaken and then incubated at 37°C. for 4 hours. By this time heavy precipitates had formed in the tubes containing Sera 3-18 and 8-3, but a less marked one in that containing Serum 3-22. All of the tubes were again shaken to suspend the precipitates and 0.5 cc. from each was mixed with 0.25 cc. of vaccine virus emulsion and injected intradermally in rabbits. The remaining portions of the mixtures were placed in the ice box and injected 2 days later into the skin of rabbits.

Additional tests were made with the antiserum-antigen mixture to test for possible differences in the action of the fluid and precipitate respectively. Those made up with Sera 3-18 and 8-3 were centrifuged, the precipitate removed and suspended in 4 cc. of Ringer's solution. To the supernatant fluid and the precipitate suspension was added half their volume of the vaccine virus suspension. 0.75 cc. of each was immediately injected intradermally in rabbits, and 0.75 cc. of the remaining mixture was also injected after 2 days in the ice box. In each case vaccine virus alone was inoculated for control. The results of these tests are shown in Table IV. This experiment was repeated 4 times with variations in the length of contact and the relative amounts of the ingredients making up the mixture, the outcome being always the same as in Table IV. An idea of the results of the 5 experiments can be gained from Text-fig. 1, which expresses graphically the general differences observed with the mixtures used.

The antiserum for rat testicle extract not only neutralizes the virus-enhancing power of rat testicle extracts, but the lesions produced by the antiserum-testicle extract-virus mixtures are even smaller than the controls. The neutralization is not a staple reaction, however, as is shown by the fact that the mixtures injected after 2 days in the ice box result in moderately enhanced lesions. No significant differences

could be observed between the lesions produced by the whole mixture, the supernatant fluid and the precipitate.

Two possible explanations of the above result suggest themselves. Either there is a direct neutralization of the enhancing factor by the antiserum, or the factor is not specifically affected but adsorbed on the flocculate which is formed by the interaction of the antiserum and



TEXT-FIG. 1. Effect of the anti-rat testicle serum on the lesions produced by vaccine virus + rat testicle extract.

the testicle extract. The following experiments are an attempt to solve this question.

*Experiment.*—The same procedure as described above was repeated, except that the mixtures of antiserum, testicle extract and virus were not incubated but were injected into rabbits immediately after being mixed. The outcome of these inoculations differed from those in the foregoing experiments in that Sera 3-18, 3-19 and 3-22, all comparatively weak in precipitins, did not inhibit the enhancing

power of the testicle extract. However, with the more powerful precipitating serum, No. 8-3, there was a definite neutralization of the action.

While the results with the weaker sera suggest that the inactivation of the testicle factor was due to adsorption on the flocculate, the more powerful serum seemed to inactivate directly. Immediate flocculation with the latter serum is a possibility to be considered.

TABLE IV

*Effect of Antiserum for Rat Testicle Extract on Infections with Vaccine Virus Enhanced by Rat Testicle Extract*

Mixture injected	Resulting lesion	Resulting lesion after mixtures had stood 2 days in ice box before injection
Anti-rat testicle Serum 3-18 + testicle extract + virus (whole mixture).....	++	+++
Anti-rat testicle Serum 3-18 + testicle extract + virus (supernatant fluid).....	+	+++
Anti-rat testicle Serum 3-18 + testicle extract + virus (precipitate).....	+±	+++
Anti-rat testicle Serum 3-22 + testicle extract + virus (whole mixture).....	++	+++
Anti-rat testicle Serum 8-3 + testicle extract + virus (supernatant fluid).....	+	
Anti-rat testicle Serum 8-3 + testicle extract + virus (precipitate).....	++	
Normal serum + testicle extract + virus.....	++++++	++++++
Ringer's solution + testicle extract + virus.....	++++++	++++++
Ringer's solution + virus.....	+++	++

The number of pluses indicates the extent of the lesion.

*Test of the Specificity of Action of Antitesticular Serum on the Enhancing and Spreading Properties of Testicle Extract*

The question is raised by the foregoing experiment as to whether the enhancing factor acts as an antigen or whether the antibodies are developed against the incidental proteins. If the sera developed against the testicle extract of one species neutralizes the spreading and enhancing factor of an extract from another, it would indicate an antibody against the factor, for this is not species-specific.

The antigen used in these experiments was a purified fraction from bull testicle. This material, developed in collaboration with Claude and Helmer by methods to be reported later, is a stable powder, very low in protein, containing in concentrated form practically all of the spreading and enhancing factor present in the full organ extract. 3 rabbits, 5-12, 5-14 and 5-17, were injected intravenously with 5 cc. of a solution of this powder, the strength of which, judged by its spreading power, is 3 to 4 times as great as that of the extract as such. The injections were repeated 5 times at 3 day intervals, and 8 days after the last injection the animal was bled for serum.

The precipitating power of the three sera was tested against the purified fraction and the full bull testicle extract, and also against the active fraction of guinea pig and rat testicle extract. The amount of serum was kept constant and the antigen progressively diluted. The results were read after the tubes had been incubated at 37°C. for 2 hours and kept overnight in the ice box. A definite precipitate occurred with both the purified and ordinary testicle extract, with a titer ranging from a dilution of 1:30 for Serum 5-14 up to 1:200 with Sera 5-12 and 5-17. There was no reaction with the guinea pig and rat testicle extracts, nor did normal rabbit serum have any precipitating power for any of the extracts.

The first tests with the sera thus produced showed that the strongest antisera completely neutralized the virus-enhancing power of bull testicle extract, a result similar to that recorded in the foregoing section for rat testicle. Using the same technique, these two sera were tested against guinea pig and rat testicle extracts. While the results were not clear-cut, there was no definite neutralization of the enhancing power.

As the spreading power for inert particles and the enhancement of infections by testicle extract seem to be manifestations of the action of the same factor, the former offered a better test for the study of the antiserum action.

In all of the experiments in this group, the purified fractions of bull, rat and guinea pig testicle extract have been used with Antisera 5-12, 5-14 and 5-17, developed against the purified bull testicle fraction. The fractions were diluted with Ringer's solution up to a point where the spreading power was approximately that of the fresh extract. Equal amounts of the antiserum and diluted testicle material were mixed, incubated for 2 hours at 37°C. and kept in the ice box overnight. Controls kept under the same conditions consisted of the extract with normal serum and with Ringer's solution. After the period of contact, the tubes were thoroughly shaken in order to suspend the precipitate. Samples of each, mixed with India ink, were injected intradermally in a rabbit. The area of spread, which could be easily followed in the shaved skin, was recorded 2 hours and 24 hours after injection.

Preliminary tests showed two points worthy of note. The neutralization of the spreading power by antisera for homologous testicle extract was most evident at the end of 2 hours, when the control with extract alone gave a spread about 5 times as great as the test material. The differences were still pronounced at the end of 24 hours; but the controls by this time were only 2 to 3 times as large. The change in ratio resulted from the fact that the mixture of antiserum and testicle extract spread at a much lower rate but continued longer, owing probably to the gradual release of some of the active fraction bound in the interaction of the antiserum and extract. The second point was that, when very concentrated solutions

TABLE V

*Effect of Antiserum for Bull Testicle Extract on the Spreading Power of Testicle Extract*

Experiment No.	Mixtures injected							
	Bull testicle extract + immune serum	Bull testicle extract + normal serum	Bull testicle extract + Ringer's solution	Rat testicle extract + immune serum	Rat testicle extract + normal serum	Rat testicle extract + Ringer's solution	Guinea pig testicle extract + immune serum	Guinea pig testicle extract + normal serum
1	1.2	2	2					
2	1	2	2					
3	1.2		4.5	6.5		2.5		
4	1		1.8					
5	1.5	3.5	2.5					
6	1.3	4						
7	1	3	2.5	2.5	3.5	2.5	2.5	2.5
8	1.2	2	2.3	1.5	2		2	2.3
9	1	3	2.5	1	1.5	1.5	1.5	1.7

The area of spread of the control injection of India ink and Ringer's solution is taken as the unit, and the spread from the other mixtures is expressed in multiples of the control.

of the testicle extracts were used, no inhibiting action of the homologous antiserum could be noted.

With the method described above, 9 experiments were carried out to contrast the action of the antisera on homologous and heterologous testicle extracts. The results are shown in Table V, in which the area of spread of the control injection of India ink and Ringer's solution is taken as the unit, and the spread from the other mixtures is expressed in multiples of the control.

The results indicate clearly that the antiserum for bull testicle ex-

tract neutralizes the spreading factor of bull testicle extract and has little or no effect upon the action of rat or guinea pig testicle extract. Possible exceptions will be noted in Experiments 8 and 9, but these can be attributed to the fact that the extracts in these instances were diluted 1:10 and 1:40 respectively. Under these conditions the normal spreading power would be reduced to a point at which it is difficult to judge differences.

*Enhancing and Spreading Power of Testicle Extract in Animals Immunized to Testicle Extract*

The *in vitro* neutralization of the spreading and enhancing properties of testicle extract by a homologous antitesticle serum has been shown in the foregoing experiments. In the next experiments the possibility of *in vivo* neutralization was tested.

This group consisted of 2 rabbits injected intravenously with rat testicle extract, 4 rabbits injected intraperitoneally with rat testicle extract and 3 receiving rooster testicle extract intravenously. While these animals did not develop precipitins in a high titer with one exception, all were sensitized, as shown by a strongly positive Arthus phenomenon. At intervals of 8 to 25 days after the completion of the series of immunizing injections, those animals which had received the rat testicle extract were inoculated in 3 areas on one flank with 0.25 cc. of vaccine virus in varying dilutions, and in the other flank with the same amounts of virus with rat testicle extract. For each test normal rabbits were inoculated with the same material as controls. The results showed that the enhancement of the infection by testicle extract was just as great in the animals immunized to testicle extract as it was in the control rabbits. Similar tests made with India ink showed that the spreading power was equally unimpaired. The 3 rabbits immunized with rooster testicle extract and injected with vaccine virus alone, developed lesions equal to those in a control animal.

It is clear that the neutralization of the testicle factor does not take place *in vivo*. This supports the supposition that the *in vitro* neutralization by the homologous antitesticle extract serum is an indirect phenomenon linked with the flocculation of the incidental proteins present, not a true neutralization in the immunological sense of the term.

DISCUSSION AND SUMMARY

The experiments reported here show that the infection-enhancing factor of testicle extract is neutralized *in vitro* by an antiserum against

homologous testicle extract. An antiserum developed against a testicle extract of one species does not influence the enhancing and spreading factor of the extract from another species. Rabbits immunized against testicle extract do not exhibit any alteration in the spreading or enhancing effect of extracts employed later, even when the testicle extract used is from the same species as that employed for the immunization.

Whether the *in vitro* inactivation of the active factor is a specific neutralization, or is the result of adsorption of the factor on the flocculate formed, has not been definitely determined. The fact that there is no neutralization *in vivo*, and that the antiserum acts only on extracts from the same species, when definite flocculation takes place, tends to emphasize the probability that the neutralization is not a direct one, but is incidental to the flocculation mentioned.

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## CORNEAL REACTIONS TO BACTERIUM GRANULOSIS AND OTHER MICROORGANISMS

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### PLATE 31

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During the course of studies on the relationship of *Bacterium granulosis* to trachoma, it has been possible to produce lesions in monkeys and apes which closely resemble the conjunctival lesions of the human disease (1-3). Pannus, considered by many ophthalmologists as indicative of trachoma, has not, however, been demonstrated in the experimental disease.

Pannus is generally regarded as a circumscribed, vasculonebulous keratitis. As defined by Cuénod and Nataf (4): Pannus tenuis (or pannus vasculosus) is a type of lesion in which delicate, new-formed blood vessels lie in a faint opacity of the corneal surface. Pannus crassus, occurring infrequently, is an opaque thickening of the corneal epithelium with more extensive, yet less evident, vascularization. Fuchs (5) describes the microscopic lesion as a "vasculo-granular" infiltration of the corneal layers above and below Bowman's membrane. There is no uniformity of opinion, however, concerning the cause of this corneal change. Moretti (6), for example, believes that pannus is the result of propagation by contiguity to the cornea of the granular reaction in the conjunctiva, the alteration in appearance being due to the different anatomical substrate of the cornea. Many ophthalmologists support the opinion that the condition is caused by mechanical irritation: the repeated rubbing of rough, granular lids over the cornea eventually leads to injury of the membrane with later lymphoid infiltration and formation of blood vessels. De Schweinitz (7) states, however, that traumatic irritation may be only a predisposing factor.

Since corneal changes play such an important rôle in trachoma, and inasmuch as similar lesions have not been reproduced experimentally, we have studied the effects of injury to the cornea by various agents. In this way we have sought to determine the type and degree of stimulus necessary to produce the delicate, vascularized, corneal opacity characteristic of trachomatous pannus.



There are certain reasons for regarding the corneae of the lower animals as more resistant to injury than the human cornea. They are exposed to eye injury from foreign bodies and other agents and yet, under natural conditions, inflammatory and destructive corneal lesions are rarely encountered in them. Even when gritty and abrasive substances or material from cages in which the animals are confined find their way into the eyes producing occasionally superficial lesions of the cornea, no severe effects follow. As Noguchi (1) and ourselves (3) have shown, repeated subconjunctival injections of cultures of organisms which are found in the conjunctival sac of monkey and man suffering from different kinds of granular conjunctivitis (3) bring about either a transient reaction or none, while repeated application of cultures of *Bacterium granulosis* to the abraded cornea is without noteworthy effect.

In the light of these facts, it is more readily understood why the animal cornea does not tend to participate in the chronic granular conjunctival inflammation which can be induced either by human trachomatous tissues or by cultures of *Bacterium granulosis*, and why it becomes essential to inject microorganisms directly into the cornea in order to study their injurious action on that tissue.<sup>1</sup>

### *Methods and Materials*

The action of the various microorganisms and of the tissue suspensions was first studied in rabbits. Then monkeys, either normal or taken during the height of experimental *granulosis* conjunctivitis, were used to study the effects of *Bacterium granulosis* after intracorneal injection.

*Intracorneal Inoculation.*—The animal was deeply anesthetized. The conjunctival sac of one eye was flushed with warm saline solution and a lid retractor inserted. The bulbar conjunctiva near the limbus was then grasped with a curved iris forceps, and with a No. 27 gauge needle the conjunctiva was penetrated and the point of the needle carried about 2 mm. beyond the limbus, into and toward the center of the cornea. From 0.05 to 0.075 cc. of suspension was gently injected into the tissue. Care was taken to avoid escape of the inoculum into the anterior chamber, in which event the results were less regular.

*Corneal Examination.*—A hand slit-lamp was used in a dark room for examination of the cornea. At times it was advantageous to anesthetize an animal so as to immobilize the eyeball.

*Bacterial Suspensions Employed.*—The inoculum consisted of a suspension of the

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<sup>1</sup> All operative procedures on animals were done with the aid of ether anesthesia.

full growth of bacteria on blood agar slants. The suspension was made with the condensation water of the medium in 2 cc. of saline solution. The bacteria inoculated intracorneally in rabbits were:

1. *Bacterium granulosis*, eight strains, one sent us from Tunis by Dr. Phillips Thygeson, two from cases of trachoma in Denver, Colorado, and Albuquerque, New Mexico, and five from New York cases.<sup>2</sup>

2. Several varieties of bacteria isolated from normal or affected conjunctivae of man and monkey were pooled. These consisted of *Streptococcus viridans*, a Gram-positive diplobacillus, a diphtheroid, *Staphylococcus albus*, *Bacillus xerosis*, a Gram-negative, spore-bearing bacterium, and a small Gram-negative, chromogenic bacillus (two types).

3. *Staphylococcus albus*, two strains, obtained from the conjunctival secretions of a monkey having experimental trachoma.

4. Minute Gram-negative bacillus recovered from the filtrate of a suspension of human trachomatous conjunctival tissue.

5. *Bacillus xerosis*, from the unfiltered material of the latter case.

6. Small, Gram-negative bacillus, motile and chromogenic, isolated from the conjunctiva of a monkey with spontaneous granular conjunctivitis; also a similar organism, recovered from a case of follicular conjunctivitis in man.

7. A Gram-negative, proteolytic, chromogenic bacillus; contaminant of the Tunisian strain of *Bacterium granulosis*.

8. *Hemophilus influenzae*, a pathogenic, meningeal strain described by Pittman as Type b (8).

9. Pneumococcus, Type I, highly pathogenic for mice, derived from lobar pneumonia in man.

10. Pneumococcus, Type II, moderately pathogenic for mice; derived from the nasopharyngeal secretions of a healthy person.

11. *Streptococcus viridans*, from normal human nasopharyngeal secretions.

12. *Streptococcus hemolyticus*, from a healthy person.

13. Gonococcus, from a case of acute bartholinitis.

14. Gram-negative, motile, chromogenic bacillus derived from a Tunisian case of human trachoma.

Most of the varieties of bacteria employed were therefore derived from the conjunctival sac of man or monkey. In addition, series of rabbits were inoculated intracorneally with sterile leptospira medium, physiological saline solution, cultures of *Bacterium granulosis* killed by heating at 56°C. for 30 minutes, tissue suspensions from a case of human trachoma, and suspensions of scrapings of tissue from a monkey having *granulosis* conjunctivitis.

<sup>2</sup> For the Western American cultures we are indebted to Dr. Rowland P. Wilson of Cairo, Egypt, and for the material yielding the New York strains to Dr. Martin Cohen, of New York City. For consultations and clinical examination of experimental animals, we wish to thank Dr. Martin Cohen and Dr. H. W. Wooton, formerly of the Trachoma Hospital, New York.

*Experimental Results in Rabbits*

*Bacterium granulosis*.—Four of twenty-five rabbits inoculated intracorneally with the Tunisian culture of *Bacterium granulosis* failed to develop corneal lesions.<sup>3</sup> With one exception (Rabbit A), to be described, the remaining twenty-one animals reacted uniformly.

During the intracorneal inoculation, a vesicle appears at the site of injection, about 3 or 4 mm. in diameter. This flattens out in a few hours, leaving a faintly greyish, wedge-shaped macula. On the following day, moderately intense congestion of the conjunctivae, ciliary injection, and diffuse, faint clouding of the upper third or half of the cornea appear. On the 3rd day the conjunctivitis and ciliary injection persist; the diffuse clouding of the cornea becomes lighter, and the wedge-shaped, greyish macula, with its base at the limbus, is less dense. At the same time a network arises of coarse, superficial vessels in the bulbar conjunctiva, tortuous and anastomosing, but in the main running in parallel lines from the fornix to the part of the limbus adjacent to the opacity just described. These vessels are seen only over a limited area, the width of which is demarcated by the extent of the base of the corneal lesion. The episcleral vessels in general are only moderately distended. On the 3rd to the 5th days, the signs of acute inflammation have subsided, while on the 6th day, the surface of the palpebral conjunctiva appears granular, the localized injection of the bulbar conjunctiva persists, and a definite clearing of the cornea occurs except at the site of inoculation. Here is seen a faint, greyish, wedge-shaped opacity, at the base of which are newly formed, superficial blood vessels arising from the conjunctival vessels at the limbus. From one to five or more interlacing superficial vessels can be seen entering the opaque region. On the 7th day, appearances reproduced in Fig. 1 are present. From now on to the 15th day, the localized vascularization proceeds actively; the anastomosing, fine vessels extend radially and terminate quite sharply at the apical end of the opacity, which is now faint and limited to the vascular zone. After 5 or 6 weeks the eye has returned practically to normal except for this zone, about 1.5 to 4 mm. at the base and about 4 to 6 mm. long. At this time the condition resembles closely pannus tenuis or pannus vasculosus of human trachoma. The lesions are still present in six of the animals 6 months after inoculation; in the remaining rabbits the corneal condition endured from 2 to 5 or more months.

The exceptional case referred to above (Rabbit A) was one in which *Staphylococcus aureus* infection was spontaneously superimposed on that of *Bacterium granulosis*. The reaction, after 7 days, as shown by Fig. 2, was much more severe, especially in respect to the granular conjunctivitis and the corneal involvement. The effect of secondary infection on *granulosis* conjunctivitis has already been described (3).

The corneal lesion at the height of the reaction shows foci of lymphoid infiltra-

<sup>3</sup> This strain was also non-pathogenic in *Macacus rhesus* monkeys.

tions with occasional plasma cells and large mononuclear cells. Numerous small, newly formed blood vessels are present in the involved areas. The main infiltration and vascularization arise between the epithelial layer and Bowman's membrane, in a space where these two layers have been forced apart. The substantia propria and the epithelium adjacent to the infiltrated areas likewise show a moderate degree of similar cellular invasion. The histology agrees well with descriptions of the changes of human pannus corneae.

In four rabbits, a second intracorneal injection in the same eye was made with cultures of *Bacterium granulosis*, and in one of them, a third and fourth. The reinoculations were made 1 to 2 months after the primary injection. The general effects of the reinoculations were much less marked than those of the first injection, and small corneal lesions endured for a period of only 1 to 3 weeks. It seems, therefore, that while complete resistance does not arise a partial immunity to later injections of *Bacterium granulosis* develops.

The fact should be pointed out that granular conjunctivitis is also produced but, because rabbits frequently exhibit this condition spontaneously and the subconjunctival injection of various substances produces it, the follicular reaction cannot be considered as specific.

*Other Microorganisms.*—The intracorneal injection of the pooled cultures of ordinary conjunctival microorganisms listed in the second group (see "Methods and materials," above) induced, after 24 hours, marked panophthalmitis in two rabbits. In one animal suppurative, destructive panophthalmitis occurred. In the other, hemorrhagic, non-granular conjunctivitis, cyclitis, iritis, and interstitial keratitis, with generalized vascularization of the cornea, and secondary glaucoma arose. In this rabbit a secondary cataract supervened 2 months later. The more pronounced residual signs after 6 months consisted of cataract, interstitial keratitis, and marked glaucoma.

*Staphylococcus albus.*—Within 24 hours after inoculation with this organism, three rabbits developed suppurative keratitis, follicular conjunctivitis, cyclitis, and iritis. 1 week later there was a narrow, circumferential vascularization of the cornea which continued to extend and to cover the entire membrane. After 6 weeks cataract formed in two animals and a corneal scar in the third. The rabbits with cataract had also a residual interstitial keratitis. Other rabbits of this series will be described later.

*Bacillus xerosis.*—24 hours after inoculation destructive changes in the external ocular tissues were demonstrable in two rabbits, and during the following 2 weeks abscess in the cornea, diffuse vascularized keratitis, secondary glaucoma, and cataract developed. The active lesions tended to subside so that the vascularization and opacity of the cornea diminished and came to be confined to the site of inoculation. Now, after 5 months, a localized vasculonebulous keratitis resembling that produced by *Bacterium granulosis* persists. Other residual effects are cataract and corneal scars.

*Gram-Negative Bacilli from Spontaneous Monkey Folliculosis and Human Folliculosis.*—Only a simple, transient, greyish white macula, about 3 mm. in

diameter at the site of inoculation was induced in two animals by the bacillus derived from a monkey. In two rabbits injected with the human organism, the only effect was persistent, circumferential arborization of fine conjunctival blood vessels at the limbus, with a few vascular branches barely penetrating the cornea. The upper one-half of the limbus was affected. Complete recovery occurred in from about a week to a month. Either a similar effect or no result whatever followed the injection of six rabbits with the Gram-negative, motile, chromogenic Tunisian bacillus described on page 125.

*Proteolytic, Chromogenic Bacillus*.—The organism induced, 24 hours after inoculation, a greenish tinged, small, localized abscess, accompanied in one of three rabbits by follicular conjunctivitis. The abscess and subsequent vascularized ulceration endured in one animal for 3 weeks, then retrogressed and healed. Other rabbits of this series will be described later in the section on dilution.

*Hemophilus influenzae*.—On the day following the injection of a culture, a diffuse, suppurative reaction set in. On the 3rd day appeared intense follicular conjunctivitis, purulent keratitis with localized abscesses, and fixation of the lens with an opacity on the anterior capsule. There was profuse, thick secretion in the conjunctival sac. Marked episcleritis and iritis were also observed. 1 month after inoculation vasculonebulous, localized keratitis was visible in one of two rabbits, which at the height of the reaction resembled the corneal change induced by *Bacterium granulosis*; but after 3 weeks it had completely disappeared.

*Pneumococcus Types I and II*.—Pneumococcus Type I was found to be lethal for rabbits after intracorneal inoculation: all nineteen animals injected died 24 to 48 hours later. At the time of death they showed active panophthalmitis. On this account a culture of Pneumococcus Type II was substituted. Within 24 hours, the culture induced at the site of inoculation an intense hemorrhagic and follicular conjunctivitis, and suppurative keratitis with localized abscess formation. After 10 days a uniformly clouded cornea with generalized, radial vascularization was observed, the vessels extending from the limbus toward the pupil. After 16 days, examination revealed characteristic localized vasculonebulous keratitis. This endured for 2 weeks, and then receded to complete disappearance in about 1 month. Other animals of the pneumococcus series will be described in the next section.

*Streptococcus viridans*.—The reaction of two animals to this organism almost paralleled the changes induced by Pneumococcus Type II.

*Streptococcus hemolyticus*.—Rapidly destructive panophthalmitis with early signs of meningoencephalitis followed the inoculation of hemolytic streptococci. Before the death of the four injected animals, streptococcal septicemia supervened.

*Gonococcus*.—24 hours after injection, an intense follicular conjunctivitis appeared, accompanied by hypopyon, suppurative keratitis and iridocyclitis, and after 10 days generalized vascularization of the cornea. Within 17 days there was noted a distinct localized vasculonebulous keratitis which endured in one rabbit for only 1 week and was present in another animal at the time it was sacrificed for microscopic studies 18 days later.

*Filtrable Gram-Negative Bacillus*.—Inoculation of a culture of this micro-organism, described on page 125, induced at the site of inoculation only a transient bleb.

*Control Materials*.—A similar evanescent bleb formation followed the intracorneal inoculation of eight rabbits with sterile leptospira medium, saline solution, or heat-killed *granulosis* cultures.

*Tissue Suspensions*.—Four rabbits were injected intracorneally with unfiltered tissue suspensions: two with the ground conjunctival tissue derived from a human case of trachoma and two with similar tissues obtained from a monkey at the height of experimental *granulosis* conjunctivitis. As one might expect from the use of material containing a number of different microorganisms (3), two of the animals, one from each series, developed destructive panophthalmitis and died shortly thereafter. The remaining two rabbits showed a clinical course similar to that of the animals inoculated with the group of ordinary conjunctival bacteria (Group 2). The employment, therefore, of such tissue inoculations in rabbits for a study of specific lesions is impracticable.

The results obtained after intracorneal inoculation of rabbits with several varieties of bacteria show a wide diversity of effects. For example, organisms such as the filtrable Gram-negative bacillus and chromogenic bacilli (or control inanimate materials) fail to induce a specific reaction. On the other hand, pathogenic species, such as *Pneumococcus* Type I, *Streptococcus hemolyticus*, and others produce destructive, suppurative lesions of the eye and its appendages. Among the latter organisms, however, are a group of bacteria, such as *Pneumococcus* Type II and *Streptococcus viridans*, which are not so highly pathogenic. They give rise to milder suppurative lesions in the cornea, accompanied by transient inflammation of the conjunctiva, sclera, ciliary body, iris, and occasionally, the lens. After subsidence of the acute inflammatory reaction, within several days to about a month, localized, vasculonebulous keratitis resembling the lesion induced by *Bacterium granulosis* remains. *Bacterium granulosis* having a particular virulence of low degree causes regularly a non-suppurative, uncomplicated, more or less enduring, localized, vasculonebulous keratitis which has a certain resemblance to the pannus tenuis of human trachoma. The degree of the corneal reaction, therefore, serves as an index of the pathogenicity of the organisms.

*Effect of Dilution*.—The question arises whether cultures of bacteria having a high degree of pathogenicity can be made by dilution to induce vascularized opacities of the cornea.

Three highly pathogenic strains, *Pneumococcus* Type I, *Staphylococcus albus*, and the Gram-negative proteolytic bacillus (Group 7) were selected. Each of these had induced highly destructive, suppurative lesions, as already noted. Dilutions of cultures were made, in the case of the first culture from 1:10 to 1:1,000,000, of the second, from 1:100 to 1:10,000, and of the third from 1:10 to 1:100. Repeated experiments were made with undiluted, standard suspensions of the cultures and with the diluted materials.

The results of tests with diluted cultures, employing 50 rabbits, are summarized as follows: With respect to *Pneumococcus* Type I, injection of dilutions up to 1:500 or 1:700 caused generalized panophthalmitis and death of the animal within 24 to 72 hours. With dilutions from about 1:700 to 1:1,600 a minute abscess 1 to 2 mm. in diameter promptly developed at the site of inoculation, which was completely resolved after 1 to 2 weeks. In animals receiving dilutions of 1:1,500 to 1:4,000 an arborization of conjunctival vessels arose about the upper third of the limbus, with fine vascular branches penetrating superficially into the cornea for a distance of never more than about 0.5 mm. The condition lasted, as a rule, for about a month. Finally, with still higher dilutions, no noteworthy effects were visible.

The intracorneal inoculation of the diluted cultures of staphylococci and of proteolytic bacilli gave rise to similar reactions: Lower dilutions caused minute abscesses with the vascular penetration of the cornea as described in the preceding paragraphs, and higher dilutions were inactive.

The results of the tests indicate, therefore, that dilutions of cultures that produce severe, destructive, suppurative changes in the rabbit's eye fail to bring about the distinctive vasculonebulous keratitis.

### *Experimental Results in Monkeys*

In view of the results obtained with *Bacterium granulosis* in rabbits, we turned next to monkeys, in which the specific conjunctival lesions due to *Bacterium granulosis* appear.

Four normal monkeys and three with experimental trachoma were each inoculated in the cornea of one eye with H. D. strain of *Bacterium granulosis*—a strain which consistently produced pannus-like lesions in the rabbit.

Two of the normal monkeys showed in from 6 to 13 days after inoculation the characteristic, small, limited, wedge-shaped keratitis similar in appearance and development to the lesion seen in rabbits. No suppurative or other involvement of the cornea was detected. After about 2 weeks, the conjunctivae of the inoculated eyes revealed congestion, edema, roughness, and a number of follicles, simulating experimental trachomatous conjunctivitis. The third monkey showed early hypopyon, episcleritis, and cyclitis. It is possible that some of the material entered the anterior chamber, under which condition these changes might have been produced; in any event, after 2 weeks, the monkey exhibited a pannus-like

lesion and characteristic granular conjunctivitis. The fourth monkey died of tuberculosis shortly after inoculation.

The three monkeys showing experimental granular conjunctivitis, which were inoculated intracorneally with the same strain of *Bacterium granulosis*, yielded similar corneal changes. In addition, the congestion and edema of the conjunctiva became more marked, showing more numerous and succulent follicles in the palpebral, as well as in the bulbar conjunctivae of both eyes (Fig. 3).

At the present time, 6 months after inoculation, three of the five animals which showed corneal lesions still have pannus-like changes, and two have completely recovered after the condition had persisted for 3 or 4 months. One animal, inoculated about a month ago, is still exhibiting localized vasculonebulous keratitis.

The two animals reported in the preceding paragraph as having recovered have been reinoculated intracorneally with the same organisms, 4 months after the original injection. Although both eyes were inoculated, in neither has any reaction been obtained. Thus, as already shown in the rabbit, a certain resistance to reinoculation can be secured in the monkey.

The direct intracorneal inoculation of *Bacterium granulosis* in *Macacus rhesus* monkeys induces a vasculonebulous keratitis which is indistinguishable from the lesion obtained in rabbits, and is similar in the degree of reaction, at least, to pannus tenuis or vasculosus of human trachoma. Furthermore, this procedure can give rise in normal monkeys to granular lids resembling the experimental trachomatous disease. In monkeys showing granular conjunctivitis before corneal injection, there is an exacerbation of the signs of the preexisting conjunctivitis.

#### DISCUSSION AND SUMMARY

The conclusions which may be drawn from the results of the experiments here presented are:

1. The cornea of the rabbit is highly sensitive to the action of various injected bacteria. The lesions vary from insignificant, transient changes to severe, destructive panophthalmitis, with fine gradations from the mildest to the violent form of inflammation. Moreover, animals that receive the same organisms show like changes.<sup>4</sup>

<sup>4</sup> As early as 1880, Councilman (9), applying caustics, such as silver nitrate or cautery, to the corneae of frogs and cats, found that the cornea "offers special advantages . . . from the comparative ease with which it can be investigated both in the fresh and prepared condition, from the facility with which inflammation *varying in extent and intensity* can be produced here and from the unity of its cellular elements."



2. The varying degree of inflammatory reaction is related to the pathogenicity of the special culture employed; as, for example, is shown by the reactions to Type I pneumococci and to *Bacterium granulosis*. It is evident that when a microorganism having a certain degree of virulence is used, a lesion of localized vasculonebulous keratitis resembling pannus tenuis or vasculosus of human trachoma can be induced. Thus *Bacterium granulosis*, *Bacillus xerosis*, *Hemophilus influenzae*, Pneumococcus Type II, *Streptococcus viridans*, and gonococcus can cause the pannus-like corneal changes in the rabbit. Of these organisms, however, only *Bacterium granulosis* induces early, uncomplicated and enduring keratitic lesions;<sup>5</sup> the others cause first, diffuse keratitis with suppurative lesions; then, as a residual effect, transient, localized, vasculonebulous changes in the cornea. These changes, in contradistinction to the *granulosis* lesions, are, therefore delayed, complicated, and transient. When, on the other hand, the invasiveness and infecting power of the organisms are low, as is the case with the filtrable, Gram-negative bacillus and the small, Gram-negative bacilli ultimately derived from cases of folliculosis, no marked effect is produced by their intracorneal inoculation. If the pathogenicity of bacteria is high (as shown by Pneumococcus Type I, hemolytic streptococcus, and the remaining bacteria), intracorneal inoculation of the microorganisms leads to serious suppurative or destructive changes.

3. The results of experiments with monkeys indicate that while pannus is not a sequel of experimental trachomatous conjunctivitis, a lesion resembling it follows intracorneal inoculation of *Bacterium granulosis*.

4. One can infer from these results, therefore, that the stimulus necessary to produce corneal changes in animals, similar to those of

<sup>5</sup> The extent of the typical *granulosis* reaction was found to be limited to the area in which the initial injection was made. Thus, if the upper sixth of the corneal surface was inoculated, only that part was involved, while if a series of injections were made around the entire margin of the cornea, the whole membrane became affected. The lesion in any one part, then, was identical with that in any other. That several of the reactions obtained were smaller than some of the lesions seen in human trachoma, appeared to be dependent only upon the size of the inoculation bleb. This condition did not hold for highly pathogenic organisms.

trachomatous pannus, is an agent having a definite but extremely low power of invasiveness and infectivity.

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#### EXPLANATION OF PLATE 31

All drawings about twice natural size.

FIG. 1. Eye of rabbit inoculated intracorneally with a culture of *Bacterium granulosis*. 7th day of reaction. Outstanding features are the congested, edematous, and roughened conjunctiva; the coarse, anastomosing vessels in the bulbar conjunctiva, and the focal vasculonebulous keratitis. The opacity of the cornea later became fainter and smaller, so that within a month the lesion resembled that shown in Fig. 3.

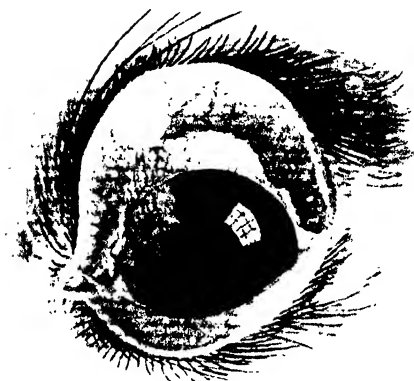
FIG. 2. The same as in Fig. 1, except that a spontaneous secondary infection with *Staphylococcus aureus* occurred. The follicular reaction in the palpebral, and the vascular lesion in the bulbar conjunctiva, as shown, are more marked.

FIG. 3. *Macacus rhesus* monkey, having experimental granular conjunctivitis as a result of subconjunctival inoculation of *Bacterium granulosis*, was injected intracorneally with this organism on the 38th day of the disease. The follicular conjunctival reaction then became more pronounced and follicles were also seen on the bulbar conjunctiva. The drawing was made about a month after the intracorneal inoculation. It shows a marked granular conjunctivitis; two small scars in the palpebral conjunctiva; coarse, anastomosing vessels in the bulbar conjunctiva, limited to the area of the corneal lesion; succulent, large follicles in the bulbar conjunctiva, and the characteristic focal, vasculonebulous keratitis.





1



2



Louis Schmidt  
-1931-

3



## CONFIGURATIONAL RELATIONSHIP OF HYDROCARBONS

### IV. OPTICAL ROTATIONS OF HYDROCARBONS OF THE ISOAMYL SERIES. THE CONFIGURATIONAL RELATIONSHIP OF SUBSTITUTED CARBONIC ACIDS CONTAINING AN ISOBUTYL AND AN ISOAMYL GROUP TO THOSE OF THE CORRESPONDING NORMAL CARBONIC ACIDS

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The present communication deals with optically active hydrocarbons of the homologous series of 1,1-methylisoamylpropanes in which the methyl and isoamyl groups remain unchanged.

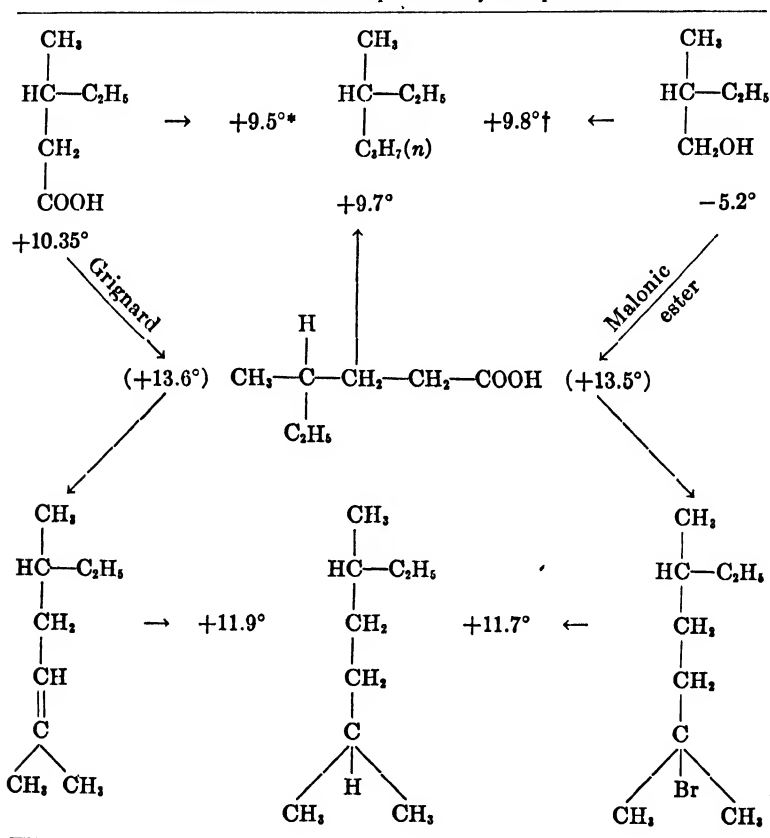
In Papers I and III<sup>1</sup> of this series it was shown that the distance of the isopropyl group from the asymmetric carbon atom was an important factor determining the direction and value of the rotation of the hydrocarbon. Thus, optically active trisubstituted methanes containing an isopropyl group rotated in the opposite direction from those containing an isobutyl group in place of the isopropyl. The lower members of the isobutyl series rotated in the same direction as the members of the normal series, their values of rotation being higher in the members of the isobutyl series. The members beyond the butylisobutyl member rotated in the opposite direction from the normal, their values being lower.

As regards the isoamyl series, two possibilities were to be considered. If distance alone played the major part in determining the effect of the isopropyl group, then the members of this series should rotate in the same direction as the members of the isobutyl series; were the even or the odd position with respect to the asymmetric carbon atom the determining factor, then the members

<sup>1</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 405 (1931); **92**, 455 (1931).

of the isoamyl series might be expected to rotate in the same direction as those of the isopropyl series, or at least might have a rotation value lower than that of the members of the normal

TABLE I  
Maximum Molecular Rotations from Experimental Data to Show No  
Racemization in Preparation of Compounds



\* Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 82 (1931).

† Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 764 (1931).

series. Indeed, the methylethylisoamylmethane previously prepared by us had a value of rotation lower than that of the corresponding member of the normal series.<sup>2</sup> However, when the

<sup>2</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 405 (1931).

higher members of the isoamyl series were prepared, it was observed that the values of their rotations approached very closely those of the members of the normal series. It was therefore considered necessary to reinvestigate the value of the rotation of the methylethylisoamylmethane. A scrutiny of every step in the preparation of the hydrocarbon aroused the suspicion that one of these steps might have been accompanied by considerable racemization; namely, the dehydration of the 2-ethyl-5-dimethylpentanol-5 which had been accomplished by means of iodine at the boiling temperature of the carbinol. The dehydration was therefore repeated with oxalic acid as a dehydrating agent. Under these conditions, the reaction was accomplished at a lower temperature, thus reducing the danger of racemization. The methylethylisoamylmethane prepared in this manner had a higher rotation; namely,  $[M]_D^{25} = +11.9^\circ$ , in place of  $+7.09^\circ$  as previously reported. In order to test the correctness of this value, the hydrocarbon was also prepared by reduction of 1-bromo-1,1-dimethyl-4-ethylpentane by shaking with palladium and hydrogen. A practically identical value was obtained for the rotation of the hydrocarbon; namely,  $[M]_D^{25} = +11.7^\circ$ . *Thus we are confident that the value of the molecular rotation of the methylethylisoamylmethane as now obtained is correct* (Table I).

*Preparation and Configurational Relationships of Higher Members of Methylethylisoamylmethane Series*—The starting materials for the higher members of this series were the configurationally related butanoic acids-4 substituted in position (2). The acids of this series have been correlated by the direct chemical method and therefore the hydrocarbons derived from them may be correlated among themselves on a reliable basis. The individual steps for the preparation of the hydrocarbons are shown in Table II. In Table II are given the configurationally related substituted butanoic acids-4 and the hydrocarbons derived from them. It may be seen that the configurationally related hydrocarbons containing the isoamyl group including the methylamylisoamylmethane rotate in the same direction. The values of rotation given in Table II are experimental, and not maximum rotations.

*Extrapolation of Maximum Rotations*—The maximum rotations were calculated by multiplying the observed values by the ratio  $\frac{\text{maximum rotation}}{\text{observed rotation}}$  of the parent substance. This mode of calculation



tion is permissible in cases where racemization is excluded. The test in the case of methylethylisoamylmethane described above justifies the assumption that the reactions leading from the butanoic acids-4 substituted in position (2) are not accompanied

TABLE II  
*Experimental Values Obtained in Preparation of Hydrocarbons Containing a Methyl and an Isoamyl Group.  $[M]_D^{25}$*

	$-\text{CH}_2-\text{COOH}$	$-\text{CH}_2-\text{CH}_2\text{Br}$	$-\text{CH}_2-\text{CH}_2-\text{COOH}$	$-\text{CH}_2-\text{CH}_2-\text{COOC}_2\text{H}_5$	$\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{CH}_2-\text{CH}=\text{C} \\ \diagup \\ \text{CH}_3 \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{CH}-\text{CH} \\ \diagup \\ \text{CH}_3 \end{array}$
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_2\text{H}_5 \end{array}$	-3.04	-6.51*	-4.02	-3.76	-3.01	-3.52
$\begin{array}{c} \text{CH}_3 \dagger \\   \\ \text{HC}- \\   \\ \text{C}_3\text{H}_7(n) \end{array}$	+1.59	-9.29	-3.04	-2.43	-1.83	-1.55
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_4\text{H}_9(n) \end{array}$	+3.14	-9.01	-2.10	-1.17	-0.91	-0.76
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_5\text{H}_{11}(n) \end{array}$	+4.56	-8.30	-1.01	-0.24	-0.59	-0.04

\* The chloride in this case was used instead of the bromide.

† For convenience of discussion, the signs of all members of this series were changed from those found experimentally.

by racemization. Even had racemization occurred, it should have been of the same order of magnitude in the entire series of derivatives so that the differences in the rotations of members of homologous series should be of approximately the same order of magnitude as in the case in which racemization had not occurred.

TABLE III\*

Maximum Molecular Rotations of Configurationally Related Hydrocarbons (in Homogeneous Solution) of the General Type  $\begin{array}{c} \text{CH}_3 \\ | \\ \text{H}-\text{C}-\text{R}_2 \\ | \\ \text{R}_1 \end{array}$

Where  $\text{R}_1$  and  $\text{R}_2$  are Alkyl Radicals.  $[M]_D^{25}$

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HCOH} \\   \\ \text{C}_2\text{H}_5 \\ +10.3 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \\ -10.35 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_2\text{H}_5 \\   \\ \text{C}_2\text{H}_5 \\ 0 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_2\text{H}_7(n) \\   \\ \text{C}_2\text{H}_5 \\ -9.9 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_4\text{H}_9(n) \\   \\ \text{C}_2\text{H}_5 \\ -11.4 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_6\text{H}_{11}(n) \\   \\ \text{C}_2\text{H}_5 \\ -12.0 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_4\text{H}_9(\text{iso}) \\   \\ \text{C}_2\text{H}_5 \\ -21.3 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_6\text{H}_{11}(\text{iso}) \\   \\ \text{C}_2\text{H}_5 \\ -11.9 \end{array}$
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HCOH} \\   \\ \text{C}_3\text{H}_7(n) \\ +12.2 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_3\text{H}_7(n) \\ +3.60 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_2\text{H}_5 \\   \\ \text{C}_3\text{H}_7(n) \\ +9.9 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_3\text{H}_7(n) \\   \\ \text{C}_3\text{H}_7(n) \\ 0 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_4\text{H}_9(n) \\   \\ \text{C}_3\text{H}_7(n) \\ -1.7 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_6\text{H}_{11}(n) \\   \\ \text{C}_3\text{H}_7(n) \\ -2.4 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_4\text{H}_9(\text{iso}) \\   \\ \text{C}_3\text{H}_7(n) \\ -14.9 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_6\text{H}_{11}(\text{iso}) \\   \\ \text{C}_3\text{H}_7(n) \\ -3.5 \end{array}$
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HCOH} \\   \\ \text{C}_4\text{H}_9(n) \\ +11.8 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_4\text{H}_9(n) \\ +6.06 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_2\text{H}_5 \\   \\ \text{C}_4\text{H}_9(n) \\ +11.4 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_3\text{H}_7(n) \\   \\ \text{C}_4\text{H}_9(n) \\ +1.5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_4\text{H}_9(n) \\   \\ \text{C}_4\text{H}_9(n) \\ 0 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_6\text{H}_{11}(n) \\   \\ \text{C}_4\text{H}_9(n) \\ -0.8 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_4\text{H}_9(\text{iso}) \\   \\ \text{C}_4\text{H}_9(n) \\ -11.9 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_6\text{H}_{11}(\text{iso}) \\   \\ \text{C}_4\text{H}_9(n) \\ -1.5 \end{array}$
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HCOH} \\   \\ \text{C}_5\text{H}_{11}(n) \\ +11.8 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_5\text{H}_{11}(n) \\ +8.12 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_2\text{H}_5 \\   \\ \text{C}_5\text{H}_{11}(n) \\ +12.5 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_3\text{H}_7(n) \\   \\ \text{C}_5\text{H}_{11}(n) \\ +2.4 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_4\text{H}_9(n) \\   \\ \text{C}_5\text{H}_{11}(n) \\ +0.8 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_6\text{H}_{11}(n) \\   \\ \text{C}_5\text{H}_{11}(n) \\ 0 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_4\text{H}_9(\text{iso}) \\   \\ \text{C}_5\text{H}_{11}(n) \\ -9.3 \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{C}_6\text{H}_{11}(\text{iso}) \\   \\ \text{C}_5\text{H}_{11}(n) \\ -0.2 \end{array}$

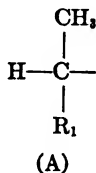
\* These values are calculated on the basis of maximum rotations of the corresponding propionic acids from which they were prepared (Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **92**, 456 (1931)).

The values of rotation of the members of the normal, isobutyl, and isoamyl series are given in Table III.

#### CONCLUSIONS

From Table III it may be seen that the members of the isoamyl series of hydrocarbons have approximately the same value of rotation as the members of the normal amyl series. Thus, it would seem that in the case of aliphatic hydrocarbons, *the isopropyl group introduces a special rotatory contribution only when located either directly on the asymmetric carbon atom or at a distance of 1 carbon only from the asymmetric carbon atom.*

A second important conclusion may be formulated from a scrutiny of the data contained in Table III. If one compares a series of homologous hydrocarbons with a corresponding series of carbinols, as, for instance, the one given in Column 1 of Table III, the following is observed: The values of the rotations in the series of carbinols progressively increase. The values of the rotations of the hydrocarbons of Column 3 likewise progressively increase and have the same sign as those of the carbinols. The values in Column 4 decrease from the first to the third member and then progressively increase, the sign having changed after the symmetric member. In Columns 5 and 6, the values of all the members above the symmetric progressively decline, the sign being opposite from that of the carbinols, whereas those of the members below the symmetric progressively increase, having the same sign as the corresponding carbinols. The values of the members of the isobutyl and of the isopropyl series progressively decline in value. All these changes in values may be explained on the assumption that the rotation of every one of these substances is composed of two major contributions of opposite sign. In a simplified way, to the radicle



the positive value A may be assigned and to the group R<sub>2</sub> the negative contribution (B). Thus, when A is greater than B, the

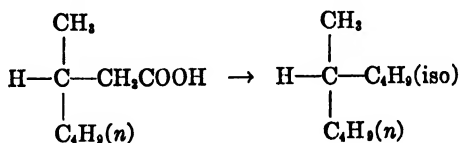
substance rotates in the direction of A, and, as the value of A increases, the value of the rotation of the substance increases. On the other hand, when the value of B is greater than that of A, then the direction of rotation of the substance is negative, and, if the value of B remains constant, A now increasing, then the values of the rotation of the substances will progressively decline. On the other hand, if the value A remains constant, the value B increasing, then the values of the substances will decline until the symmetric member is reached and will increase beyond that member. Such a condition is observed in the horizontal rows of Table III.

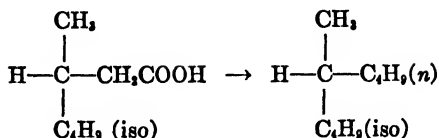
Returning now to the isobutyl and isoamyl series, it is evident that, in the light of the above discussion, *the isobutyl group has a higher negative value than the normal butyl, whereas the isoamyl group has only a slightly higher value than the corresponding normal group.*

We wish to emphasize, however, that in speaking of the contribution of any one group we do not wish to attribute to it an independent value, but a value which is itself the result of the vicinal effect of all the other groups. This applies equally to groups A and B. We also wish to emphasize that the value of group B in every vertical column remains only approximately constant.

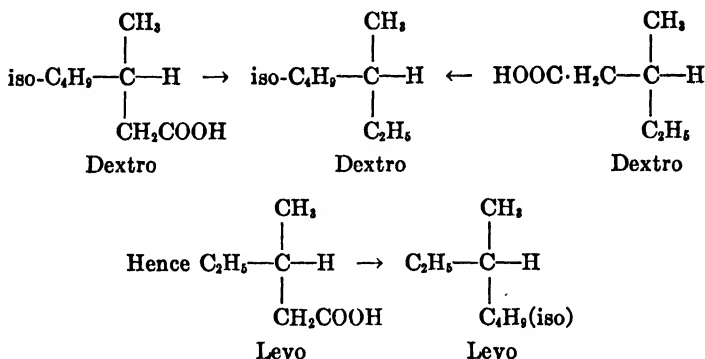
*Configurational Relationship of Substituted Carbonic Acids Containing an Isobutyl and an Isoamyl Group to Those of Corresponding Carbonic Acids Containing a Normal Butyl or a Normal Amyl Group*

Since the configurations of the hydrocarbons with respect to the disubstituted carbonic acids have been established, it has now become possible to correlate the configurations of the above two types of carbonic acids. From the following formulæ it may be seen that the two acids which lead to enantiomorphous hydrocar-

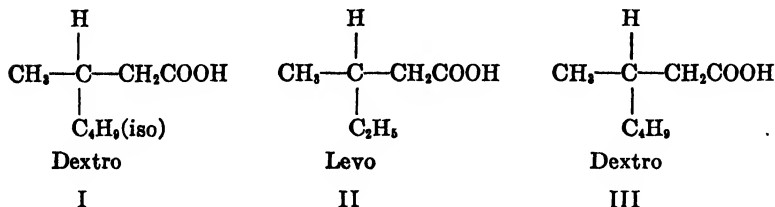




bons are configurationally related. The following were the reactions actually performed.

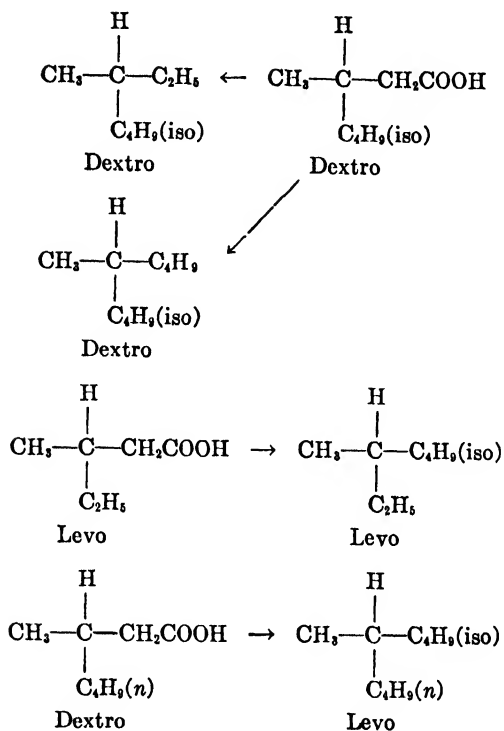


From this it follows that dextro-2-isobutylbutanoic acid-4 is configurationally related to levo-2-ethylbutanoic acid-4. This means that the following two acids may be regarded as configurationally related, inasmuch as in projection the isobutyl and the ethyl groups are located on the same side of the butanoic acid when they rotate in opposite directions.



Furthermore, since levo-2-ethylbutanoic acid-4 is configurationally related to dextro-2-butylbutanoic acid-4, it follows that 2-isobutylbutanoic acid-4 and 2-butylbutanoic acid-4 are configurationally related when they rotate in the same direction. It is also evident that 2-butylbutanoic acid-4 and 2-isobutylbutanoic acid-4 rotating in

the same direction will lead to hydrocarbons rotating in opposite directions.



The configurational relationship of the 2-isoamylbutanoic acid-4 to the corresponding normal acid was established in the same way, inasmuch as dextro-2-ethylbutanoic acid-4 and dextro-2-isoamylbutanoic acid-4 both lead to dextro-methylethylisoamylmethane.

*Maximum Rotations of 2-Isobutyl- and of 2-Isoamylbutanoic Acids-4*—The original task in the preparation of these two acids was their resolution to the maximum rotations with the object of preparing the hydrocarbons of the isobutyl and of the isoamyl series by methods which would definitely exclude the possibility of racemization. Unfortunately, the resolution of these two acids proved a very difficult task. However, the knowledge of the so called maximum rotations of the hydrocarbons of the isobutyl and of the isoamyl series makes it possible to calculate a value for the

rotations of 2-isobutyl and of 2-isoamylbutanoic acids-4. These values then permit of an estimate of the effect on the rotation of substituting the butyl group by an isobutyl and the amyl by an isoamyl. Thus, multiplying the experimental value for the molecular rotation of 2-isobutylbutanoic acid-4 by the ratio  $\frac{21.7}{3.4}$  a

TABLE IV

*Comparison of Configurational Relationship of Isobutyl and Isoamyl Derivatives to n-Butyl and n-Amyl Derivatives*

Experimental results (not maximum rotations).  $[\text{M}]_D^{25}$ .

	-CH <sub>2</sub> -COOH	-CH <sub>2</sub> -COOC <sub>2</sub> H <sub>5</sub>	-CH <sub>2</sub> -CH <sub>2</sub> OH	-CH <sub>2</sub> -CH <sub>2</sub> Br	-CH <sub>2</sub> -CH <sub>2</sub> -CH <sub>2</sub> OH	-C <sub>2</sub> H <sub>5</sub>	-C <sub>2</sub> H <sub>5</sub> (n)
$\begin{array}{c} \text{CH}_3^* \\   \\ \text{HC}- \\   \\ \text{C}_4\text{H}_9(n) \end{array}$	+5.5	+2.6	+3.6	-15.0	+0.7	+10.3	+1.4
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_4\text{H}_9(\text{iso}) \end{array}$	+2.4	+1.9	+1.9	-1.0		+3.4	
$\begin{array}{c} \text{CH}_3^* \\   \\ \text{HC}- \\   \\ \text{C}_5\text{H}_{11}(n) \end{array}$	+7.1	+3.7	+5.4	-13.0	+2.3	+11.0	+2.1
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_5\text{H}_{11}(\text{iso}) \end{array}$	+3.8	+2.6	+3.2	-4.6	+1.3	+5.4	+1.4

\* Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

value of 15.0° for the rotation of the acid is obtained, whereas the corresponding normal acid has a rotation of 6.0°. The 2-isoamylbutanoic acid-4, on the same principle, has a maximum molecular rotation of  $\frac{3.8 \times 11.9}{-5.4} = 8.40^\circ$  against a value of 8.1°

for the corresponding normal acid. Thus, the substitution of a normal butyl group by an isobutyl group enhances the rotation considerably. The substitution of a normal amyl group by an isoamyl group has little effect on the rotation (see Table IV).

#### EXPERIMENTAL

*Dextro-2-Ethylvaleric Acid-5*—A Grignard reagent was prepared from 8 gm. of magnesium in 200 cc. of dry ether and 40 gm. of 1-chloro-3-methylpentane,  $[\text{M}]_D^{25} = +6.51^\circ$  (from 2-ethylbutyric acid-4,  $[\text{M}]_D^{25} = +3.04^\circ$ ). The solution was cooled and dry carbon dioxide passed into it for about 30 minutes. The Grignard solution was decomposed in the usual way, and the ether distilled. The acid was purified through its sodium salt. This was then decomposed and the organic acid distilled. B.p.  $115^\circ$  at 16 mm. Yield 29 gm.

$$[\alpha]_D^{22} = \frac{+2.85^\circ}{1 \times 0.923} = +3.09^\circ; [\text{M}]_D^{22} = +4.02^\circ \text{ (homogeneous)}$$

Calculated maximum  $[\text{M}]_D^{22} = +13.59^\circ$  (homogeneous).

The maximum rotation calculated from the 2-ethylbutyric acid-4 was in agreement with that previously obtained from 1-bromo-2-methylbutane and malonic ester.<sup>3</sup>

2.848 mg. substance: 6.700 mg.  $\text{CO}_2$  and 2.838 mg.  $\text{H}_2\text{O}$   
 $\text{C}_7\text{H}_{14}\text{O}_2$ . Calculated. C 64.6, H 10.8  
 Found. " 64.2, " 11.2

*Dextro-Ethyl Ester of 2-Ethylvaleric Acid-5*—59 gm. of 2-ethylvaleric acid-5,  $[\text{M}]_D^{22} = +4.02^\circ$ , were dissolved in 150 cc. of absolute alcohol, and 5 cc. of concentrated sulfuric acid were added. The product was refluxed on a steam bath for  $\frac{1}{2}$  hour, the excess alcohol distilled off under reduced pressure, water added, and the ester extracted with ether. It was then distilled. B.p.  $80^\circ$  at 20 mm. Yield 60 gm.  $D \frac{22}{4} = 0.888$ .

$$[\alpha]_D^{22} = \frac{+2.11^\circ}{1 \times 0.888} = +2.38^\circ; [\text{M}]_D^{22} = +3.76^\circ \text{ (homogeneous)}$$

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<sup>3</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 79 (1931).



3.277 mg. substance: 8.185 mg. CO<sub>2</sub> and 3.389 mg. H<sub>2</sub>O  
 C<sub>9</sub>H<sub>18</sub>O<sub>2</sub>. Calculated. C 68.3, H 11.5  
 Found. " 68.1, " 11.6

*Dextro-4-Methyl-1-Hexanol*—50 gm. of ethyl ester of 3-methylcaproic acid-6,  $[M]_D^{25} = +2.60^\circ$ , were dissolved in 200 cc. of absolute alcohol and then reduced by dropping into a suspension of 90 gm. of sodium in 450 cc. of toluene with rapid stirring. The carbinol was isolated and purified as previously described. B.p. 77° at 20 mm. Yield 31 gm.  $D_{\frac{28}{4}} = 0.818$ .

$$[\alpha]_D^{25} = \frac{+1.79^\circ}{1 \times 0.818} = +2.19^\circ; [M]_D^{25} = +2.54^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{28} = +11.9^\circ$  (homogeneous).

3.930 mg. substance: 10.430 mg. CO<sub>2</sub> and 4.930 mg. H<sub>2</sub>O  
 C<sub>7</sub>H<sub>16</sub>O. Calculated. C 72.3, H 13.9  
 Found. " 72.1, " 14.0

*Dextro-4-Methyl-1-Bromohexane*—100 gm. of phosphorus tribromide were slowly added to 62 gm. of 4-methyl-1-hexanol,  $[M]_D^{28} = +2.54^\circ$ . The product was heated on a steam bath for 15 minutes, poured onto ice, and the halide extracted with ether. The ether was evaporated and the bromide purified in the usual way. B.p. 78° at 44 mm. Yield 65 gm.

$$[\alpha]_D^{25} = \frac{+2.95^\circ}{1 \times 1.129} = +2.61^\circ; [M]_D^{28} = +4.67^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{28} = +21.9^\circ$  (homogeneous).

5.835 mg. substance: 10.060 mg. CO<sub>2</sub> and 4.520 mg. H<sub>2</sub>O  
 C<sub>7</sub>H<sub>15</sub>Br. Calculated. C 46.9, H 8.5  
 Found. " 47.0, " 8.6

*Dextro-Methylethylpropylmethane*—A Grignard reagent was prepared from 25 gm. of 4-methyl-1-bromohexane,  $[M]_D^{28} = +2.44^\circ$ , and 3 gm. of magnesium in 50 cc. of dry ether. The ethereal solution was poured onto ice and the hydrocarbon extracted with ether. The ether was distilled. The hydrocarbon was shaken with cold concentrated sulfuric acid, then with sodium carbonate, and

finally washed with water. It was dried with sodium sulfate and then distilled from sodium. B.p.  $92^{\circ}$  at 760 mm. Yield 4 gm.

$$D \frac{28}{4} = 0.681.$$

$$[\alpha]_D^{25} = \frac{+1.12^{\circ}}{1 \times 0.681} = +1.64^{\circ}; [M]_D^{25} = +1.65^{\circ} \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{28} = +9.67^{\circ}$  (homogeneous).

4.714 mg. substance: 14.555 mg.  $\text{CO}_2$  and 6.686 mg.  $\text{H}_2\text{O}$

$\text{C}_7\text{H}_{16}$ . Calculated. C 83.9, H 16.1

Found. " 84.2, " 15.9

*Dextro-Methylethylisoamylmethane*—60 gm. of ethyl ester of 2-ethylvaleric acid-5,  $[M]_D^{22} = +3.76^{\circ}$ , were added to 1 mol of methylmagnesium iodide in dry ether. The Grignard solution was poured onto ice and ammonium chloride solution and the carbinol was extracted with ether. The ether solution was dried with anhydrous sodium sulfate; then the ether was distilled off under reduced pressure. The carbinol was not distilled.

30 gm. of crude carbinol were mixed with 10 gm. of oxalic acid and slowly distilled at atmospheric pressure. The operation was repeated. The distillate was separated from the water, dried with sodium sulfate, and the unsaturated hydrocarbon distilled. B.p.  $138^{\circ}$  at 760 mm. Yield 17 gm.

$$[\alpha]_D^{25} = \frac{+1.77^{\circ}}{1 \times 0.740} = +2.39^{\circ}; [M]_D^{25} = +3.02^{\circ} \text{ (homogeneous)}$$

(a) To 10 gm. of the unsaturated hydrocarbon was added 1 gm. of platonic oxide. Reduction was carried out by shaking with hydrogen under a pressure of 30 pounds per square inch. The reduction was complete in 15 minutes. The hydrocarbon was shaken with cold, concentrated sulfuric acid, washed with water and sodium carbonate solution, dried with sodium sulfate, then distilled from a small piece of sodium. B.p.  $134^{\circ}$  at 760 mm.

Yield 8 gm.  $D \frac{22}{4} = 0.717$ .

$$[\alpha]_D^{25} = \frac{+1.97^{\circ}}{1 \times 0.717} = +2.75^{\circ}; [M]_D^{25} = +3.52^{\circ} \text{ (homogeneous)}$$

4.376 mg. substance: 13.561 mg. CO<sub>2</sub> and 6.105 mg. H<sub>2</sub>O  
 C<sub>9</sub>H<sub>10</sub>. Calculated. C 84.3, H 15.7  
 Found. " 84.5, " 15.6

(b) 20 gm. of the carbinol were shaken with 200 cc. of cold hydrobromic acid. This was warmed on a steam bath for  $\frac{1}{2}$  hour. The oil which separated was distilled. B.p. 92° at 20 mm. Yield 24 gm. This was placed in a flask with 100 cc. of 10 per cent sodium hydroxide and 1 gm. of colloidal palladium. It was shaken with hydrogen for 24 hours. The hydrocarbon was extracted with ether and distilled. It was purified as previously described, then redistilled from sodium. B.p. 134° at 760 mm. Yield 5 gm.

$$[\alpha]_D^{25} = \frac{+1.95^\circ}{1 \times 0.717} = +2.72^\circ; [M]_D^{25} = +3.48^\circ \text{ (homogeneous)}$$

3.999 mg. substance: 12.352 mg. CO<sub>2</sub> and 5.610 mg. H<sub>2</sub>O  
 C<sub>9</sub>H<sub>10</sub>. Calculated. C 84.3, H 15.7  
 Found. " 84.2, " 15.7

*Dextro-2-Propylvaleric Acid-5*—A Grignard reagent was prepared from 6 gm. of magnesium in 100 cc. of dry ether and 45 gm. of 1-bromo-3-methylhexane,  $[M]_D^{21} = +9.29^\circ$  (from 2-propylbutyric acid-4,  $[\alpha]_D^{25} = -1.22^\circ$ ). Dry carbon dioxide was passed for  $\frac{1}{2}$  hour into the cooled Grignard solution. The product was poured onto ice and hydrochloric acid, and the organic acid extracted with ether. It was then distilled. B.p. 132° at 22 mm. Yield 31 gm.  $D \frac{24}{4} = 0.882$ .

$$[\alpha]_D^{25} = \frac{+1.86^\circ}{1 \times 0.882} = +2.11^\circ; [M]_D^{24} = +3.04^\circ \text{ (homogeneous)}$$

3.475 mg. substance: 8.525 mg. CO<sub>2</sub> and 3.500 mg. H<sub>2</sub>O  
 C<sub>9</sub>H<sub>10</sub>O<sub>2</sub>. Calculated. C 66.6, H 11.2  
 Found. " 66.9, " 11.3

*Dextro-Ethyl Ester of 2-Propylvaleric Acid-5*—31 gm. of 2-propylvaleric acid-5,  $[M]_D^{25} = +3.04^\circ$ , were dissolved in 100 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid were added. This was refluxed  $\frac{1}{2}$  hour on a steam bath. The excess alcohol was distilled off under reduced pressure, water was added, and the

ester extracted with ether. The solution was dried with sodium sulfate, then distilled. B.p. 104° at 32 mm. Yield 33 gm.

$$D \frac{24}{4} = 0.859.$$

$$[\alpha]_D^{24} = \frac{+1.21^\circ}{1 \times 0.859} = +1.41^\circ; [M]_D^{24} = +2.43^\circ \text{ (homogeneous)}$$

3.685 mg. substance: 9.505 mg. CO<sub>2</sub> and 4.000 mg. H<sub>2</sub>O

C<sub>10</sub>H<sub>20</sub>O<sub>2</sub>. Calculated. C 69.7, H 11.7

Found. " 70.3, " 12.1

*Dextro-Methyl-n-Propylisoamylmethane*—33 gm. of ethyl ester of 2-propylvaleric acid-5,  $[M]_D^{24} = +2.43^\circ$ , were added to 0.5 mol of methylmagnesium iodide in dry ether. The Grignard solution was decomposed by ice and ammonium chloride solution and the carbinol extracted with ether. The ether was distilled and the residue distilled from 12 gm. of oxalic acid. The unsaturated hydrocarbon was separated from the water, dried, and distilled from sodium. B.p. 162°. Yield 14 gm.

$$[\alpha]_D^{24} = \frac{+0.98^\circ}{1 \times 0.750} = +1.31^\circ; [M]_D^{24} = +1.83^\circ \text{ (homogeneous)}$$

To 14 gm. of the unsaturated hydrocarbon was added 1 gm. of platonic oxide. The hydrocarbon was reduced by shaking with hydrogen under a pressure of 30 pounds per square inch. The reduction was complete in 15 minutes. The hydrocarbon was shaken with cold concentrated sulfuric acid, then washed with water and sodium carbonate solution, dried with sodium sulfate, and distilled from sodium. B.p. 156° at 760 mm. Yield 12 gm.

$$D \frac{25}{4} = 0.725.$$

$$[\alpha]_D^{25} = \frac{+0.79^\circ}{1 \times 0.725} = +1.09^\circ; [\alpha]_D^{25} = +1.55^\circ \text{ (homogeneous)}$$

4.412 mg. substance: 13.675 mg. CO<sub>2</sub> and 6.200 mg. H<sub>2</sub>O

C<sub>11</sub>H<sub>22</sub>. Calculated. C 84.4, H 15.6

Found. " 84.5, " 15.7

*Levo-2-n-Butylvaleric Acid-5*—A Grignard reagent was prepared from 6 gm. of magnesium in 100 cc. of dry ether and 50 gm. of 1-bromo-3-methylheptane,  $[M]_D^{24} = -9.01^\circ$  (from 2-butylbutyric

acid-4,  $[\alpha]_D^{25} = +2.18^\circ$ ). This was cooled in ice and carbon dioxide passed in for  $\frac{1}{2}$  hour. The Grignard solution was poured onto ice and hydrochloric acid. The organic acid was extracted with ether, the ether evaporated, and the acid purified by extracting an aqueous solution of its sodium salt with ether. The acid was then distilled. B.p.  $149^\circ$  at 22 mm. Yield 33 gm.  $D \frac{25}{4} = 0.871$ .

$$[\alpha]_D^{25} = \frac{-1.17^\circ}{1 \times 0.871} = -1.34^\circ; [M]_D^{25} = -2.13^\circ \text{ (homogeneous)}$$

3.191 mg. substance: 7.935 mg.  $\text{CO}_2$  and 3.320 mg.  $\text{H}_2\text{O}$

$\text{C}_9\text{H}_{18}\text{O}_2$ . Calculated. C 68.3, H 11.5

Found. " 67.8, " 11.6

*Levo-Ethyl Ester of 2-n-Butylvaleric Acid-5*—33 gm. of 2-n-butylvaleric acid-5,  $[M]_D^{25} = -2.10^\circ$ , were mixed with 100 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid added. The product was refluxed on a steam bath for  $\frac{1}{2}$  hour, then the excess alcohol was distilled off under reduced pressure. Water was added and the ester extracted with ether. It was then distilled.

B.p.  $112^\circ$  at 23 mm. Yield 36 gm.  $D \frac{25}{4} = 0.861$ .

$$[\alpha]_D^{25} = \frac{-0.55^\circ}{1 \times 0.861} = -0.64^\circ; [M]_D^{25} = -1.19^\circ \text{ (homogeneous)}$$

2.779 mg. substance: 7.200 mg.  $\text{CO}_2$  and 3.055 mg.  $\text{H}_2\text{O}$

$\text{C}_{11}\text{H}_{22}\text{O}_2$ . Calculated. C 70.9, H 11.9

Found. " 70.7, " 12.3

*Levo-Methyl-n-Butylisoamylmethane*—To 1 mol of methylmagnesium iodide in ether were added 36 gm. of ethyl ester of 2-n-butylvaleric acid-5,  $[M]_D^{25} = -1.17^\circ$ . The Grignard solution was poured onto ice and ammonium chloride, and the carbinol was extracted with ether. The ether was evaporated under reduced pressure and the residue heated at  $150^\circ$  for  $\frac{1}{2}$  hour with 20 gm. of oxalic acid. The unsaturated hydrocarbon was then distilled under reduced pressure and finally from sodium. B.p.  $113^\circ$  at 100 mm. Yield 9 gm.

$$[\alpha]_D^{25} = \frac{-0.44^\circ}{1 \times 0.759} = -0.58^\circ; [M]_D^{25} = -0.89^\circ \text{ (homogeneous)}$$

9 gm. of the unsaturated hydrocarbon were placed in a bottle with 1 gm. of platonic oxide and reduced by shaking with hydrogen under 30 pounds per square inch pressure. The hydrocarbon was purified as described for methylethylisoamylmethane. B.p.  $109^{\circ}$  at 100 mm. Yield 7 gm.  $D \frac{25}{4} = 0.738$ .

$$[\alpha]_D^{25} = \frac{-0.36^{\circ}}{1 \times 0.738} = -0.49^{\circ}; [M]_D^{25} = -0.76^{\circ} \text{ (homogeneous)}$$

4.341 mg. substance: 13.420 mg.  $\text{CO}_2$  and 5.970 mg.  $\text{H}_2\text{O}$

$\text{C}_{11}\text{H}_{24}$ . Calculated. C 84.5, H 15.5

Found. " 84.3, " 15.4

*Levo-n-Amylvaleric Acid-5*—A Grignard reagent was prepared from 52 gm. of 1-bromo-3-methyloctane,  $[M]_D^{24} = -8.30^{\circ}$  (from 2-amylobutyric acid-4,  $[\alpha]_D^{25} = +2.92^{\circ}$ ). Carbon dioxide was passed for  $\frac{1}{2}$  hour into the Grignard solution cooled in ice. The reaction product was poured onto ice and hydrochloric acid, and the organic acid was extracted with ether. The ether was distilled off and the sodium salt of the acid was purified by extracting its aqueous solution with ether. The acid was then distilled. B.p.  $156^{\circ}$  at 22 mm. Yield 37 gm.  $D \frac{25}{4} = 0.871$ .

$$[\alpha]_D^{25} = \frac{-0.52^{\circ}}{1 \times 0.871} = -0.60^{\circ}; [M]_D^{25} = -1.03^{\circ} \text{ (homogeneous)}$$

3.975 mg. substance: 10.135 mg.  $\text{CO}_2$  and 4.145 mg.  $\text{H}_2\text{O}$

$\text{C}_{10}\text{H}_{20}\text{O}_2$ . Calculated. C 69.7, H 11.7

Found. " 69.5, " 11.7

*Levo-Ethyl Ester of 2-n-Amylvaleric Acid-5*—37 gm. of 2-n-amylobutyric acid-5,  $[M]_D^{25} = -1.01^{\circ}$ , were added to 100 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid. The mixture was heated  $\frac{1}{2}$  hour on a steam bath, and then the excess alcohol was distilled off under reduced pressure. Water was added and the ester extracted with ether. It was then distilled. B.p.  $120^{\circ}$  at 22 mm. Yield 41 gm.  $D \frac{25}{4} = 0.862$ .

$$[\alpha]_D^{25} = \frac{-0.20^{\circ}}{2 \times 0.862} = -0.12^{\circ}; [M]_D^{25} = -0.23^{\circ} \text{ (homogeneous)}$$

2.452 mg. substance: 6.440 mg. CO<sub>2</sub> and 2.685 mg. H<sub>2</sub>O  
 C<sub>12</sub>H<sub>24</sub>O<sub>2</sub>. Calculated. C 71.9, H 12.1  
 Found. " 71.6, " 12.2

*Levo-Methyl-n-Amylisoamylmethane*—41 gm. of ethyl ester of 2-*n*-amylvaleric acid-5,  $[M]_D^{25} = -0.24^\circ$ , were added to 1 mol of methylmagnesium iodide in 300 cc. of dry ether. The Grignard solution was poured onto ice and ammonium chloride solution. The carbinol was extracted with ether and the ether distilled. The residue was heated at 150° for  $\frac{1}{2}$  hour with 25 gm. of oxalic acid, then distilled under reduced pressure. The unsaturated hydrocarbon was separated from the water, dried, and distilled from sodium. B.p. 125° at 100 mm. Yield 21 gm.

$$[\alpha]_D^{25} = \frac{-0.27^\circ}{1 \times 0.766} = -0.35^\circ; [M]_D^{25} = -0.59^\circ \text{ (homogeneous)}$$

14 gm. of the unsaturated hydrocarbon were placed in a flask with 1 gm. of platonic oxide and reduced by shaking with hydrogen under 30 pounds per square inch pressure. The hydrocarbon was purified as described for methylethylisoamylmethane. B.p. 122° at 100 mm. Yield 12 gm.  $D \frac{25}{4} = 0.739$ .

$$[\alpha]_D^{25} = \frac{-0.04^\circ}{1 \times 0.739} = -0.05^\circ; [M]_D^{25} = -0.09^\circ \text{ (homogeneous)}$$

4.986 mg. substance: 15.474 mg. CO<sub>2</sub> and 6.895 mg. H<sub>2</sub>O  
 C<sub>12</sub>H<sub>24</sub>. Calculated. C 84.6, H 15.4  
 Found. " 84.6, " 15.5

*Resolution of 2-n-Butylbutyric Acid-4*—This acid was resolved by crystallizing its quinine salt from acetone at -15° until the rotation of the acid reached a constant value. This was after the eighth crystallization.

$$[\alpha]_D^{25} = \frac{-3.81^\circ}{1 \times 0.905} = -4.21^\circ; [M]_D^{25} = -6.06^\circ \text{ (homogeneous)}$$

4.885 mg. substance: 11.945 mg. CO<sub>2</sub> and 4.950 mg. H<sub>2</sub>O  
 C<sub>8</sub>H<sub>16</sub>O<sub>2</sub>. Calculated. C 66.6, H 11.2  
 Found. " 66.7, " 11.3

*Resolution of 2-n-Amylbutyric Acid-4*—This acid was resolved by crystallizing its quinine salt from acetone at  $-15^{\circ}$  until the rotation of the free acid reached a constant value. This was reached on the eighth crystallization.

$$[\alpha]_D^{27} = \frac{-4.61^{\circ}}{1 \times 0.896} = -5.14^{\circ}; [M]_D^{27} = -8.12^{\circ} \text{ (homogeneous)}$$

5.964 mg. substance: 15.025 mg.  $\text{CO}_2$  and 6.145 mg.  $\text{H}_2\text{O}$

$\text{C}_9\text{H}_{18}\text{O}_2$ . Calculated. C 68.3, H 11.5

Found. " 68.7, " 11.5

*Levo-4-Methylnonane*—A Grignard reagent was prepared from 3 gm. of magnesium in 50 cc. of dry ether and 22 gm. of 1-bromo-4-methylnonane,  $[M]_D^{27} = +5.57^{\circ}$ .<sup>3</sup> The Grignard solution was decomposed with ice and hydrochloric acid. The hydrocarbon was extracted with ether and, after the usual purification, was distilled from sodium. B.p.  $76^{\circ}$  at 30 mm. Yield 5 gm.  $D \frac{27}{4} = 0.726$ .

$$[\alpha]_D^{27} = \frac{-1.13^{\circ}}{1 \times 0.726} = -1.56^{\circ}; [M]_D^{27} = -2.21^{\circ} \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{27} = -2.47^{\circ}$  (homogeneous).

2.450 mg. substance: 7.560 mg.  $\text{CO}_2$  and 3.430 mg.  $\text{H}_2\text{O}$

$\text{C}_{10}\text{H}_{22}$ . Calculated. C 84.4, H 15.6

Found. " 84.1, " 15.7

*Dextro-2-Isobutylbutyric Acid-4*—The inactive acid was prepared from methylisobutylbromomethane and malonic ester.

1 mol of the inactive acid was dissolved in 400 cc. of boiling acetone and 1 mol of quinine was added. The solution was then filtered and 100 cc. of water added. The solution was allowed to stand in a refrigerator at  $-15^{\circ}$  until crystallization took place. The salt was filtered and recrystallized three times from 80 per cent acetone. It is very soluble and crystallizes with difficulty. For these experiments the mother liquors from the first crystallization were used. The acetone was evaporated and the quinine salt decomposed with hydrochloric acid. The organic



acid was extracted with ether, purified through its sodium salt and then distilled. B.p.  $124^{\circ}$  at 20 mm.  $D \frac{30}{4} = 0.899$ .

$$[\alpha]_D^{30} = \frac{+1.48^{\circ}}{1 \times 0.899} = +1.65^{\circ}; [M]_D^{30} = +2.37^{\circ} \text{ (homogeneous)}$$

3.881 mg. substance: 9.433 mg.  $\text{CO}_2$  and 4.000 mg.  $\text{H}_2\text{O}$

$\text{C}_8\text{H}_{16}\text{O}_2$ . Calculated. C 66.6, H 11.2

Found. " 66.3, " 11.5

*Dextro-Ethyl Ester of 2-Isobutylbutyric Acid-4*—100 gm. of 2-isobutylbutyric acid-4,  $[M]_D^{30} = +2.37^{\circ}$  (homogeneous), were dissolved in 200 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid added. The product was heated  $\frac{1}{2}$  hour on the steam bath, then the excess alcohol was distilled. Water was added and the ester extracted with ether. The ether extract was shaken with dilute sodium carbonate, then dried, and fractionated. B.p.  $85^{\circ}$  at 20 mm. Yield 105 gm.  $D \frac{30}{4} = 0.856$ .

$$[\alpha]_D^{30} = \frac{+0.97^{\circ}}{1 \times 0.856} = +1.13^{\circ}; [M]_D^{30} = +1.95^{\circ} \text{ (homogeneous)}$$

3.675 mg. substance: 9.410 mg.  $\text{CO}_2$  and 3.890 mg.  $\text{H}_2\text{O}$

$\text{C}_{10}\text{H}_{20}\text{O}_2$ . Calculated. C 69.7, H 11.7

Found. " 69.8, " 11.8

*Dextro-3,5-Dimethylhexanol-1*—50 gm. of ethyl ester of 2-isobutylbutyric acid-4,  $[M]_D^{30} = +1.94^{\circ}$  (homogeneous), were dissolved in 200 cc. of absolute alcohol and the solution slowly dropped into a suspension of 100 gm. of sodium in boiling toluene with vigorous stirring. The excess sodium was destroyed by additional absolute alcohol. The solution was poured onto ice and the carbinol extracted with ether, dried, and distilled. B.p.  $105^{\circ}$  at 45 mm.

Yield 30 gm.  $D \frac{30}{4} = 0.815$ .

$$[\alpha]_D^{30} = \frac{+1.19^{\circ}}{1 \times 0.815} = +1.46^{\circ}; [M]_D^{30} = +1.90^{\circ} \text{ (homogeneous)}$$

3.334 mg. substance: 8.934 mg.  $\text{CO}_2$  and 4.225 mg.  $\text{H}_2\text{O}$

$\text{C}_8\text{H}_{18}\text{O}$ . Calculated. C 73.8, H 13.9

Found. " 73.1, " 14.2

*Levo-1-Bromo-3,5-Dimethylhexane*—60 gm. of 3,5-dimethylhexanol-1,  $[M]_D^{30} = +1.90^\circ$  (homogeneous), were cooled in ice and 100 gm. of phosphorus tribromide slowly added. The product was heated on a steam bath for  $\frac{1}{2}$  hour, poured onto ice, and the halide extracted with ether. The ether was distilled off and the residue treated with cold sulfuric acid to remove any unchanged carbinol. It was separated, washed with dilute sodium carbonate solution, then water. It was dried and distilled. B.p.  $91^\circ$  at 45 mm. Yield 60 gm.  $D \frac{30}{4} = 1.099$ .

$$[\alpha]_D^{20} = \frac{-0.55^\circ}{1 \times 1.099} = -0.50^\circ; [M]_D^{20} = -0.97^\circ \text{ (homogeneous)}$$

5.632 mg. substance: 10.326 mg.  $\text{CO}_2$  and 4.518 mg.  $\text{H}_2\text{O}$

$\text{C}_8\text{H}_{17}\text{Br}$ . Calculated. C 49.7, H 8.9

Found. " 50.0, " 9.0

*Dextro-3,5-Dimethylhexane*—A Grignard reagent was prepared from 30 gm. of 1-bromo-3,5-dimethylhexane,  $[M]_D^{30} = -0.97^\circ$  (homogeneous), and 3 gm. of magnesium in 100 cc. of dry ether. This was poured onto ice and hydrochloric acid. The hydrocarbon was extracted with ether, dried, and the ether distilled. The residue in the distilling flask was treated as previously described for the purification of hydrocarbons. B.p.  $111\text{--}112^\circ$  at 760 mm. Yield 10 gm.  $D \frac{30}{4} = 0.696$ .

$$[\alpha]_D^{20} = \frac{+2.08^\circ}{1 \times 0.696} = +2.99^\circ; [M]_D^{20} = +3.41^\circ \text{ (homogeneous)}$$

4.146 mg. substance: 12.817 mg.  $\text{CO}_2$  and 5.847 mg.  $\text{H}_2\text{O}$

$\text{C}_8\text{H}_{18}$ . Calculated. C 84.1, H 15.9

Found. " 84.2, " 15.8

*Dextro-2-Isoamylbutyric Acid-4*—The inactive acid was prepared from methylisoamylbromomethane and ethyl malonate.

1 mol of the acid was dissolved in 500 cc. of hot acetone and 1 mol of quinine added. When the quinine had dissolved completely, 500 cc. of water were added and the solution cooled at  $-15^\circ$  until crystallization set in. The resolution was very difficult, owing to the solubility of the quinine salt. A better result was

obtained by making use of the filtrate from the first crystallization than by attempting the recrystallization of the levo form. The acetone was evaporated from the filtrate and the quinine salt decomposed with hydrochloric acid. The organic acid was extracted with ether, purified through its sodium salt, then distilled. B.p.

140° at 25 mm.  $D \frac{26}{4} = 0.901$ .

$$[\alpha]_D^{25} = \frac{+2.20^\circ}{1 \times 0.901} = +2.44^\circ; [M]_D^{25} = +3.86^\circ \text{ (homogeneous)}$$

4.637 mg. substance: 11.675 mg. CO<sub>2</sub> and 4.665 mg. H<sub>2</sub>O

C<sub>9</sub>H<sub>18</sub>O<sub>2</sub>. Calculated. C 58.3, H 11.5

Found. " 68.6, " 11.3

*Dextro-Ethyl Ester of 2-Isoamylbutyric Acid-4*—To 60 gm. of 2-isoamylbutyric acid-4,  $[\alpha]_D^{25} = +2.44^\circ$ , were added 200 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid. The mixture was heated  $\frac{1}{2}$  hour on a steam bath, the excess alcohol distilled off, and the ester extracted with ether. The ester was then distilled. B.p. 112° at 30 mm. Yield 62 gm.  $D \frac{25}{4} = 0.862$ .

$$[\alpha]_D^{25} = \frac{+1.20^\circ}{1 \times 0.862} = +1.39^\circ; [M]_D^{25} = +2.59^\circ \text{ (homogeneous)}$$

3.705 mg. substance: 9.625 mg. CO<sub>2</sub> and 4.040 mg. H<sub>2</sub>O

C<sub>11</sub>H<sub>22</sub>O<sub>2</sub>. Calculated. C 70.9, H 11.9

Found. " 70.8, " 12.0

*Dextro-2,5-Dimethylheptanol-7*—62 gm. of ethyl ester of 2-isoamylbutyric acid-4,  $[\alpha]_D^{25} = +1.39^\circ$ , were dissolved in 200 cc. of absolute ethyl alcohol and reduced by slowly dropping into a suspension of 100 gm. of sodium in 500 cc. of boiling toluene with rapid stirring. The carbinol was isolated as previously described.

B.p. 102° at 18 mm. Yield 38 gm.  $D \frac{27}{4} = 0.823$ .

$$[\alpha]_D^{27} = \frac{+1.82^\circ}{1 \times 0.823} = +2.21^\circ; [M]_D^{27} = +3.18^\circ \text{ (homogeneous)}$$

3.461 mg. substance: 9.400 mg. CO<sub>2</sub> and 4.396 mg. H<sub>2</sub>O

C<sub>9</sub>H<sub>20</sub>O. Calculated. C 74.9, H 14.0

Found. " 74.1, " 14.2

*Levo-7-Bromo-2,5-Dimethylheptane*—38 gm. of 2,5-dimethylheptanol-7,  $[\alpha]_D^{27} = +2.21^\circ$ , were cooled in ice and 100 gm. of phosphorus tribromide were added. The mixture was heated on a steam bath for 1 hour, poured onto ice, and the halide extracted with ether. The ether was distilled and the halide, purified by the usual method, was distilled. B.p.  $108^\circ$  at 25 mm. Yield 42 gm.  $D \frac{27}{4} = 1.090$ .

$$[\alpha]_D^{27} = \frac{-2.40^\circ}{1 \times 1.090} = -2.20^\circ; [M]_D^{27} = -4.56^\circ \text{ (homogeneous)}$$

5.096 mg. substance: 9.766 mg.  $\text{CO}_2$  and 4.165 mg.  $\text{H}_2\text{O}$

$\text{C}_9\text{H}_{19}\text{Br}$ . Calculated. C 52.2, H 9.2

Found. " 52.3, " 9.1

*Dextro-2,5-Dimethylheptane*—A Grignard reagent was prepared from 3 gm. of magnesium in dry ether and 20 gm. of 7-bromo-2,5-dimethylheptane,  $[\alpha]_D^{27} = -2.20^\circ$ . This was poured into water and the hydrocarbon extracted with ether, purified, and then distilled from sodium. B.p.  $134^\circ$  at 760 mm. Yield 4 gm.

$$[\alpha]_D^{27} = \frac{+2.99^\circ}{1 \times 0.713} = +4.19^\circ; [M]_D^{27} = +5.37^\circ \text{ (homogeneous)}$$

2.682 mg. substance: 8.299 mg.  $\text{CO}_2$  and 3.800 mg.  $\text{H}_2\text{O}$

$\text{C}_9\text{H}_{20}$ . Calculated. C 84.3, H 15.7

Found. " 84.4, " 15.8

*Dextro-2,5-Dimethyloctanol-8*—A Grignard reagent was prepared from 7 gm. of magnesium in ether and 60 gm. of 7-bromo-2,5-dimethylheptane,  $[\alpha]_D^{27} = -2.20^\circ$ . To this were added 12 gm. of paraformaldehyde and the solution allowed to stand overnight. It was then poured onto ice and the carbinol extracted with ether, then distilled. B.p.  $115^\circ$  at 15 mm. Yield 31 gm.  $D \frac{27}{4} = 0.824$ .

$$[\alpha]_D^{27} = \frac{+0.67^\circ}{1 \times 0.824} = +0.81^\circ; [M]_D^{27} = +1.29^\circ \text{ (homogeneous)}$$

3.472 mg. substance: 9.674 mg.  $\text{CO}_2$  and 4.320 mg.  $\text{H}_2\text{O}$

$\text{C}_{10}\text{H}_{22}\text{O}$ . Calculated. C 75.9, H 14.0

Found. " 76.0, " 13.9

*Dextro-2,5-Dimethyloctane*—31 gm. of 2,5-dimethyloctanol-8,  $[\alpha]_D^{27} = +0.81^\circ$ , were brominated by phosphorus tribromide as described for 7-bromo-2,5-dimethylheptane. This was not purified further than by distillation, due to the limited quantity.

A Grignard reagent was prepared from 3 gm. of magnesium in dry ether and 26 gm. of the above halide. This was poured onto ice and the hydrocarbon extracted with ether. It was purified as described for 2,5-dimethylheptane. It was then distilled from a small piece of sodium. B.p.  $156^\circ$ , at 760 mm. Yield 4 gm.

$$[\alpha]_D^{28} = \frac{+0.74^\circ}{1 \times 0.723} = +1.02^\circ; [M]_D^{28} = +1.45^\circ \text{ (homogeneous)}$$

3.932 mg. substance: 12.143 mg.  $\text{CO}_2$  and 5.442 mg.  $\text{H}_2\text{O}$

$\text{C}_{10}\text{H}_{22}$ . Calculated. C 84.4, H 15.6

Found. " 84.2, " 15.5

## ON WALDEN INVERSION

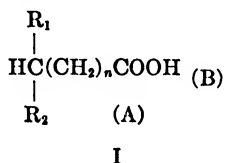
### XVII. OPTICAL ROTATIONS IN HOMOLOGOUS SERIES OF CARBOXYLIC ACIDS

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The present communication deals with the optical rotation of a series of disubstituted carbonic acids of the type

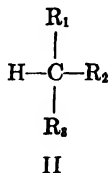


Group  $R_1$  was kept constant in the entire series of substances examined. Group  $R_2$  was a normal alkyl radicle which was progressively increased in weight. The subscript  $n$  remained constant in every vertical series and increased progressively in the horizontal series. Thus, series were obtained in which the members changed with respect to one group only. In Table I the vertical columns represent a homologous series with respect to group  $R_2$ ; the horizontal rows, a series homologous with respect to the group— $(CH_2)COOH$ .

In previous communications,<sup>1</sup> observations were reported permitting of the conclusion that the optical rotation of each substance may be regarded as if it were the resultant of two major contributions. The data presented in the present communication deal with the quantitative changes in the rotations of series homologous to one radicle only, thus showing the effect on the total rotation of the change in value of a single contribution.

<sup>1</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77, 405, 687, 761 (1931); **92**, 455 (1931); **93**, 749 (1931); **95**, 1 (1932).

In the case of normal hydrocarbons of the type of trisubstituted methanes, the two heavier groups should furnish contributions of opposite sign. Thus in the series



an increase in weight of  $R_3$  should bring about an increase of the molecular rotation towards the right whereas an increase in weight of  $R_2$  should have the opposite effect. The observations reported in previous communications are in harmony with this expectation. When neither  $R_2$  nor  $R_3$  is a simple alkyl radicle, a prediction is impossible since the contribution of any group depends upon its structure and consequently it may function as a lighter or as a heavier group than an alkyl group of the same weight. However, taking a single substance (II) and forming from it two homologous series, one with respect to  $R_3$  and the other with respect to  $R_2$ , it may be possible to arrive at a conclusion as to the sign of the contribution of each group and also as to the relative value of each group. Thus, if  $[M] = A + B$  and if  $A$  has a higher value than  $B$ , the direction of the rotation is determined by that of the contribution  $A$ . Then the values of the rotations of the consecutive members of the series homologous with respect to  $A$  should progressively increase. If the value of  $B$  is greater than  $A$ , the direction of rotation is determined by that of the contribution  $B$ . In this case, if a series is formed homologous to  $A$ , the changes in the rotations of the members of the homologous series should be in the direction of  $A$ , although the values may progressively diminish. Thus, if  $A$  is positive and  $B$  is negative and of a higher value than  $A$ , the substance should rotate to the left but as  $A$  progressively increases in weight,  $B$  remaining approximately constant, the values of the negative rotations of the successive members of the homologous series should progressively decrease, thus indicating a change towards the right.

The theoretical implications here described are illustrated in Table I. In Table I the values of the rotations given for the

TABLE I  
Maximum Molecular Rotations of Configurationally Related Aliphatic Acids Containing a Methyl Group on the Asymmetric Carbon Atom  $[M]_D^{25}$

(1)	(2)	(3)	(4)	(5)
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$ - 18.0*	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_3\text{H}_7 \end{array}$ - 10.4	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$ - 13.6	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$ - 11.1	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_2\text{H}_5 \end{array}$ - 12.2
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \\   \\ \text{C}_2\text{H}_7 \text{ (n)} \\ \text{Levo} \end{array}$ 3.6	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_3\text{H}_7 \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_3\text{H}_7 \text{ (n)} \end{array}$ - 6.9	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_3\text{H}_7 \text{ (n)} \end{array}$ - 3.7	
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \\   \\ \text{C}_4\text{H}_9 \text{ (n)} \\ \text{Levo} \end{array}$ + 6.1	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_4\text{H}_9 \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_4\text{H}_9 \text{ (n)} \end{array}$ - 4.1	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_4\text{H}_9 \text{ (n)} \end{array}$ - 1.7	
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{COOH} \\   \\ \text{C}_5\text{H}_{11} \text{ (n)} \\ \text{Levo} \end{array}$ + 8.1	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{COOH} \\   \\ \text{C}_5\text{H}_{11} \text{ (n)} \end{array}$	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_5\text{H}_{11} \text{ (n)} \end{array}$ - 1.9	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{C}_5\text{H}_{11} \text{ (n)} \end{array}$ - 0.6	

\* Marckwald, W., *Ber. chem. Ges.*, **37**, 1045, 1048 (1904).



members of the first vertical series are not the maximum values whereas those of all other members are maximum values. A comparative analysis of the second vertical series may serve as a key to the character of each of the two contributions in all the substances presented in Table I. The rotation of the first member is negative and that of all successive members is increasingly positive, indicating that the contribution of the heavier alkyl radicle is positive and that of the group containing the carboxyl is negative. In the first member the direction of rotation is determined by the  $-(\text{CH}_2)\text{COOH}$  group; in the remaining members by that of the heavier alkyl radicle. Assuming then that the direction of the contribution A remains constant for all these configurationally related substances, the conclusion is reached that in all the substances enumerated in Table I the contribution B is levorotatory. In the substances of Column 1, the value of B is higher than that of A, hence all the members rotate to the left. In Column 2 the negative value of B is smaller than in Column 1 so that it determines the direction of rotation of the first member only. In Column 3 the value of B is higher than A, so that all members rotate to the left but the rotations progressively change towards the right because of the increase in the dextrorotation of the contribution A. Finally, in Column 4 the value of B is still higher than that of A but not to the same extent as for the members of Column 3; hence the values of rotation of the individual members are lower than those of the corresponding members of Column 3 and again the change of rotation of the individual members is progressively towards the right.

Comparing the members of any *horizontal row*, one observes an alternating change in the values of the rotations of individual members. Thus it seems as if the carboxyl group attached directly to the asymmetric carbon atom furnishes a higher negative contribution than the corresponding group of the second horizontal member. The third member furnishes a higher contribution than the second and a lower than the fourth, thus the contributions are  $1 > 2 < 3 > 4$ . Thus, the effect of distance upon the contribution of a polar group is to change the value only and not the sign.

In Table II are given the maximum rotations of certain derivatives of the disubstituted propionic acids; namely, the disubstituted ethylcarbinols and disubstituted propyl bromides. In the series of these substances given in Columns 2, 3, and 4, the values of the rotations increase progressively to the right. On the basis of these observations it is possible to predict that in every other homologous series derived from the radicles given in Column 1, the values will progressively increase towards the right. Now the

TABLE II  
*Effect on Rotation of Polarity of Substituting Groups  $[M]_D^{25}$*

(1)	$-\text{CH}_2\text{COOH}$ (2)	$-\text{CH}_2\text{CH}_2\text{OH}$ (3)	$-\text{CH}_2\text{CH}_2\text{Br}$ (4)	(5)
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_2\text{H}_5 \end{array}$	-10.4	-9.2	-27.8	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_2\text{H}_5 \end{array} \quad +10.3$
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_3\text{H}_7 (n) \end{array}$	+3.6	+2.1	-21.3	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_3\text{H}_7 (n) \end{array} \quad +12.1$
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_4\text{H}_9 (n) \end{array}$	+6.1	+4.0	-16.8	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_4\text{H}_9 (n) \end{array} \quad +11.8$
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}- \\   \\ \text{C}_5\text{H}_{11} (n) \end{array}$	+8.0	+6.1	-14.7	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_5\text{H}_{11} (n) \end{array} \quad +12.0$

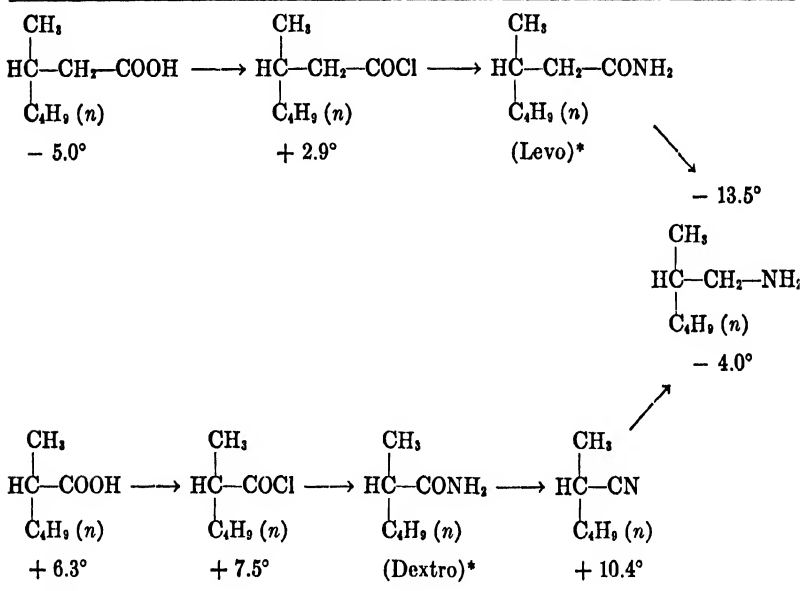
dextrorotatory secondary carbinols of the methyl series are known to possess the configuration given in Column 5. The first member of this series has a lower dextrorotation than the succeeding members. It is assumed that they are configurationally related to the other substances given in Tables I and II. On the basis of these considerations, the configuration given in Column 1 of Tables I and II was assigned as well to all the other substances given in these tables.

Thus the concept that the rotation of a given substance is

influenced by two contributions furnishes a method by which the configurations of two substances can be correlated provided there are available or are obtainable data regarding the maximum rotations of the two respective homologous series to which the substances belong. Hence it should be possible to correlate the halides derived from the radicles given in Column 1 if data on the maximum rotations of the members of the homologous series were available.

TABLE III

*Correlation of Methyl-n-Butylpropionic Acid with Methyl-n-Butylacetic Acid*



\* In alcohol.

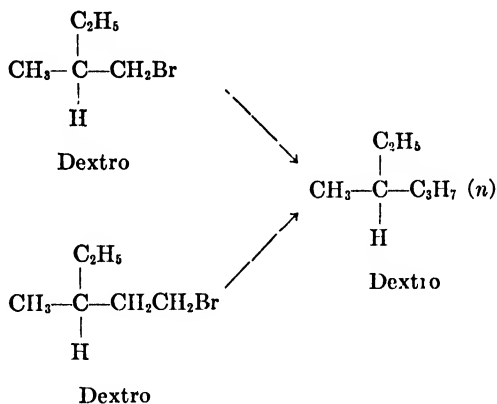
It is desired to emphasize again that the contribution of each group is not an independent property characteristic for any particular group, but is the resultant of the vicinal effect of all other groups; hence the contributions of the invariable groups in each homologous series are not absolutely constant. However, their values change in a degree which is minor as compared with that of the change introduced by the variable group.

*Preparation of the Series of Disubstituted Carbonic Acids*

The starting materials for the acids given in Table I were the corresponding disubstituted propionic acids given in Column 2 of that table. These acids have been resolved to the maximum rotations. They were converted into the corresponding carbinols which were, in turn, converted into the bromides. The maximum rotations of the derived substances were calculated on the basis of the maximum rotations of the disubstituted propionic acids.

*Configurational Relationship of Members of the Series of Disubstituted Acetic and Propionic Acids*

The configurational relationship existing between the higher members of the horizontal series derived from the 1,1-disubstituted propionic acids is self-evident and needs no further discussion. The configuration of methylethylacetic acid has been correlated previously with that of 1-methyl-1-ethylpropionic acid-3 on the basis of the following reaction.



On the basis of the set of reactions given in Table III, the same conclusion has now been reached for the higher members.

## EXPERIMENTAL

*Levo-2-n-Propylhexanoic Acid-6*—6 gm. of sodium were dissolved in 100 cc. of absolute alcohol and 40 gm. of ethylmalonate were added. To this were added 40 gm. of 1-bromo-3-methylhexane,  $[\text{M}]_D^{25} = -3.17^\circ$ . The product was refluxed with stirring for 3

hours, poured into water, and the ester extracted with ether. The ether was distilled off and the ester hydrolyzed by boiling with 60 gm. of potassium hydroxide in 80 per cent alcohol. The alcohol was evaporated and water added. The aqueous solution was acidified with hydrochloric acid and extracted with ether. The ether was evaporated and the residue heated under atmospheric pressure at  $180^{\circ}$  in a metal bath until carbon dioxide ceased to be evolved. The acid was then distilled and purified through its sodium salt. B.p.,  $127^{\circ}$  at 5 mm.; yield, 24 gm.;  $D_{\frac{25}{4}} = 0.901$ .

$$[\alpha]_D^{25} = \frac{-0.66^{\circ}}{2 \times 0.901} = -0.37^{\circ}. \quad [M]_D^{25} = -0.58^{\circ} \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = -3.67^{\circ}$  (homogeneous).

3.316 mg. substance: 8.320 mg.  $\text{CO}_2$  and 3.370 mg.  $\text{H}_2\text{O}$

$\text{C}_9\text{H}_{18}\text{O}_2$ . Calculated. C 68.3, H 11.5

Found. " 68.4, " 11.4

*Levo-2-n-Butylhexanoic Acid-6*—6 gm. of sodium were dissolved in 100 cc. of absolute alcohol and 40 gm. of ethylmalonate were added. To this were added 32 gm. of 1-bromo-3-methylheptane,  $[M]_D^{25} = -9.01^{\circ}$ . The acid was isolated and purified as described for 2-propylhexanoic acid-6. B.p.,  $130^{\circ}$  at 3 mm.; yield, 22 gm.;  $D_{\frac{25}{4}} = 0.897$ .

$$[\alpha]_D^{25} = \frac{-0.90^{\circ}}{2 \times 0.897} = -0.50^{\circ}. \quad [M]_D^{25} = -0.86^{\circ} \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = -1.6^{\circ}$  (homogeneous).

4.944 mg. substance: 12.685 mg.  $\text{CO}_2$  and 5.275 mg.  $\text{H}_2\text{O}$

$\text{C}_{10}\text{H}_{20}\text{O}_2$ . Calculated. C 69.7, H 11.7

Found. " 69.9, " 11.9

*Levo-2-n-Amylhexanoic Acid-6*—6 gm. of sodium were dissolved in 100 cc. of absolute alcohol and 40 gm. of ethylmalonate were added. To this were added 34 gm. of 1-bromo-3-methyloctane,  $[M]_D^{25} = -8.30^{\circ}$ . The acid was prepared and purified as described for 2-propylhexanoic acid-6. B.p.,  $135^{\circ}$  at 3 mm.; yield, 26 gm.;  $D_{\frac{25}{4}} = 0.893$ .

$$[\alpha]_D^{25} = \frac{-0.32^\circ}{2 \times 0.893} = -0.18^\circ. \quad [M]_D^{25} = -0.33^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = -0.6^\circ$  (homogeneous).

4.180 mg. substance: 10.835 mg.  $\text{CO}_2$  and 4.480 mg.  $\text{H}_2\text{O}$

$\text{C}_{11}\text{H}_{22}\text{O}_2$ . Calculated. C 70.9, H 11.9

Found. " 70.7, " 12.0

*Dextro-3-Methylhexanoic Acid-6*—A Grignard reagent was prepared from 8 gm. of magnesium in 200 cc. of dry ether and 40 gm. of 1-chloro-3-methylpentane,  $[M]_D^{25} = +6.51^\circ$ . Carbon dioxide was passed into the cooled solution for 30 minutes. The Grignard solution was decomposed by pouring onto ice and hydrochloric acid. The organic acid was extracted with ether, and then purified through its sodium salt. B.p.,  $115^\circ$  at 16 mm.; yield, 29 gm.;  $D \frac{22}{4} = 0.923$ .

$$[\alpha]_D^{25} = \frac{+2.85^\circ}{1 \times 0.923} = +3.09^\circ. \quad [M]_D^{25} = +4.02^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = +13.6^\circ$  (homogeneous).

2.848 mg. substance: 6.700 mg.  $\text{CO}_2$  and 2.838 mg.  $\text{H}_2\text{O}$

$\text{C}_7\text{H}_{14}\text{O}_2$ . Calculated. C 64.6, H 10.8

Found. " 64.2, " 11.1

*Dextro-4-Methylheptanoic Acid-7*—This acid was prepared by passing carbon dioxide into a Grignard reagent formed from 45 gm. of 1-bromo-3-methylhexane,  $[M]_D^{21} = +9.29^\circ$ , and 6 gm. of magnesium. It was purified as described for 3-methylhexanoic acid-6. B.p.,  $132^\circ$  at 22 mm.; yield, 31 gm.;  $D \frac{24}{4} = 0.882$ .

$$[\alpha]_D^{24} = \frac{+1.86^\circ}{1 \times 0.882} = +2.11^\circ. \quad [M]_D^{24} = +3.04^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{24} = +6.9^\circ$  (homogeneous).

3.475 mg. substance: 8.525 mg.  $\text{CO}_2$  and 3.500 mg.  $\text{H}_2\text{O}$

$\text{C}_8\text{H}_{16}\text{O}_2$ . Calculated. C 66.6, H 11.2

Found. " 66.9, " 11.3

*Levo-5-Methyloctanoic Acid-8*—The acid was prepared by passing carbon dioxide into a Grignard reagent formed from 50 gm. of 1-bromo-3-methylheptane,  $[M]_D^{24} = -9.01^\circ$ , and 6 gm. of magnesium in ether. The acid was extracted and purified as described for 3-methylhexanoic acid-6. B.p.,  $149^\circ$  at 22 mm.; yield, 33 gm.;  $D \frac{25}{4} = 0.871$ .

$$[\alpha]_D^{25} = \frac{-1.17^\circ}{1 \times 0.871} = -1.33^\circ. \quad [M]_D^{25} = -2.10^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = -4.1^\circ$  (homogeneous).

3.191 mg. substance: 7.935 mg.  $\text{CO}_2$  and 3.320 mg.  $\text{H}_2\text{O}$   
 $\text{C}_9\text{H}_{18}\text{O}_2$ . Calculated. C 68.3, H 11.7  
 Found. " 67.8, " 11.6

*Levo-6-Methylnonanoic Acid-9*—This acid was prepared by passing carbon dioxide into a Grignard reagent formed from 52 gm. of 1-bromo-3-methyloctane,  $[M]_D^{24} = -8.30^\circ$ . The acid was isolated and purified as described for 3-methylhexanoic acid-6. B.p.,  $156^\circ$  at 22 mm.; yield, 37 gm.;  $D \frac{25}{4} = 0.871$ .

$$[\alpha]_D^{25} = \frac{-0.52^\circ}{1 \times 0.871} = -0.59^\circ. \quad [M]_D^{25} = -1.01^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = -1.9^\circ$  (homogeneous).

3.975 mg. substance: 10.135 mg.  $\text{CO}_2$  and 4.145 mg.  $\text{H}_2\text{O}$   
 $\text{C}_{10}\text{H}_{20}\text{O}_2$ . Calculated. C 69.7, H 11.7  
 Found. " 69.5, " 11.7

*Dextro-3-Methylheptanoic Acid-7*—A Grignard reagent was prepared from 4 gm. of magnesium in dry ether and 30 gm. of 1-bromo-4-methylhexane,  $[M]_D^{25} = +4.67^\circ$ . Dry carbon dioxide was passed for 15 minutes into the cooled Grignard solution. The product was decomposed by means of ice and the acid purified through its sodium salt. B.p.,  $128^\circ$  at 20 mm.; yield, 12 gm.;  $D \frac{26}{4} = 0.893$ .

$$[\alpha]_D^{26} = \frac{+2.21^\circ}{1 \times 0.893} = +2.47^\circ. \quad [M]_D^{26} = +3.56^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{26} = +11.07^\circ$  (homogeneous).

5.220 mg. substance: 12.765 mg.  $\text{CO}_2$  and 5.150 mg.  $\text{H}_2\text{O}$

$\text{C}_8\text{H}_{16}\text{O}_2$ . Calculated. C 66.6, H 11.2

Found. " 66.7, " 11.0

*Dextro-3-Methyloctanoic Acid-8*—6 gm. of sodium were dissolved in 100 cc. of absolute alcohol and 40 gm. of ethylmalonate were added. To this were added 30 gm. of 4-methyl-1-bromohexane,  $[M]_D^{25} = +4.67^\circ$ . The product was boiled with stirring for 2 hours, then the ester was isolated and hydrolyzed with potassium hydroxide. The salt was extracted with ether, then acidified, and the substituted malonic acid extracted with ether. The ether was distilled off and the acid heated to  $190^\circ$  in a metal bath until carbon dioxide ceased to be evolved. It was then distilled. B.p.,  $139^\circ$  at 20 mm.; yield, 18 gm.;  $D_{\frac{25}{4}} = 0.899$ .

$$[\alpha]_D^{25} = \frac{+2.24^\circ}{1 \times 0.899} = +2.49^\circ. \quad [M]_D^{25} = +3.93^\circ \text{ (homogeneous)}$$

Calculated maximum  $[M]_D^{25} = +12.22^\circ$  (homogeneous).

4.853 mg. substance: 12.139 mg.  $\text{CO}_2$  and 5.005 mg.  $\text{H}_2\text{O}$

$\text{C}_9\text{H}_{18}\text{O}_2$ . Calculated. C 68.3, H 11.5

Found. " 68.2, " 11.5

*Levo-1-Amino-2-Methylhexane*—50 gm. of 2-butylbutyric acid-4,  $[\alpha]_D^{23} = -3.46^\circ$  (homogeneous), were refluxed on a steam bath for 15 minutes with an excess of thionyl chloride. The excess thionyl chloride was then distilled off and the residue dropped slowly into 200 cc. of cold aqueous ammonium hydroxide. The amide was filtered off, recrystallized from 50 per cent alcohol, and dried in a vacuum desiccator over sulfuric acid.

The amide was mixed with 55 gm. of bromine (1 mol) and the mixture poured into 500 cc. of 20 per cent potassium hydroxide previously heated to  $70^\circ$ . It was allowed to stand on a steam bath 2 hours, cooled, and the amine extracted with ether. The ether extract was shaken with dilute hydrochloric acid and then the aqueous layer was extracted several times with ether to remove impurities. The aqueous layer was made alkaline with potassium hydroxide solution and the amine was extracted with ether, dried



with sodium sulfate, and distilled. B.p., 62° at 22 mm.; yield 11 gm.;  $D_{\frac{27}{4}} = 0.773$ .

$$[\alpha]_D^{27} = \frac{-9.08^\circ}{1 \times 0.773} = -11.75^\circ. \quad [M]_D^{27} = -13.51^\circ \text{ (homogeneous)}$$

4.465 mg. substance: 11.863 mg. CO<sub>2</sub> and 5.860 mg. H<sub>2</sub>O

C<sub>7</sub>H<sub>17</sub>N. Calculated. C 72.9, H 14.8

Found. " 72.5, " 14.7

## CONCENTRATION OF VITAMINS B<sub>1</sub> AND B<sub>2</sub>

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The present communication contains a report on the results of several years work on the problem of the concentration of vitamin B, an investigation which was, however, discontinued by us in the spring of 1930. A review of the literature on the subject will not be given here, inasmuch as it may be found in many publications of other writers and particularly in the excellent monograph by Sherman and Smith.<sup>1</sup>

*Normal Growth of White Rat at Different Periods of the Year—* In the course of the present investigation it was observed on several occasions that the growth curve produced by a given sample of material could not be duplicated. In particular, indications were obtained suggesting that the identical material seemed to produce better growth early in the fall than later in the year. These observations caused us to undertake the study of growth from month to month, produced by a normal diet. Each new experiment was initiated on the 1st day of each month.

Four animals weighing 50.0 gm. were taken for each experiment and the weights of the animals were observed on the last day of each month. The results are given in Table I.

From Table I it may be seen that the rates of growth for the months of July and August were abnormally low, whereas the rate for September was abnormally high. The climatic conditions of New York during these 2 summer months afford an explanation of this abnormally low growth. It is possible that the abnormally high growth for September is due to a compensatory mechanism, inasmuch as the average increase in weight (52 gm.) per month

<sup>1</sup> Sherman, H. C., and Smith, S. L., The vitamins, 2nd edition, New York (1931).

for the 5 exceptional months is the same as the average increase per year.

On the basis of these findings the rate of growth of the experimental animals when placed on the vitamin diet was compared not with the average rate of growth per year but with the normal growth for the month of the experiment.

TABLE I  
*Increase in Weight on Normal Diet at Different Seasons*

Month	Rat 1	Rat 2	Rat 3	Rat 4	Average increase
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Sept.	76	89	85	89	85
Oct.	58	59	62	58	59
Nov.	59	60	58	58	59
Dec.	53	53	51	53	53
Jan.	55	53	54	56	57
Feb.	46	45	47	48	47
Mar.	49	49	47	47	48
Apr.	48	46	45	44	46
May	55	59	59	59	58
June	46	54	56	54	53
July	34	30	53	34	33
Aug.	24	25	25	24	25
Average per month for year.....					52
" " " " July, Aug.....					29
" " " " " Sept., Oct.....					72.0
" " " " " " Nov.....					59.0

*Relationship between Growth and Quantity of Dried Yeast Added to Standard Diet*—Brewers' yeast<sup>2</sup> was the material used for the preparation of vitamin extracts.

The activity of the acetone precipitate from dry yeast was used as a standard for comparison with that of the various extracts and concentrates. Only those fractions that were capable not only of maintaining normal growth but also of producing growth above normal were considered to contain all the factors present

<sup>2</sup> The yeast was furnished to us by the Jacob Ruppert Brewery. We take this occasion to express our appreciation to the management for their courtesy.

in the dry yeast, inasmuch as dry yeast possessed this faculty if given in sufficient doses.

This capacity is illustrated in Table II.

Thus, the rate of growth in October as compared with the normal was 1.4:1 in 24 days on 0.300 gm. of dry yeast per day and 1.7:1 on 0.400 gm. In April, on addition of 0.300 gm., the rate of growth as compared with the normal was 1.5:1.

TABLE II

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of Yeast*

Month	Time	Normal increase	Yeast added per day		
			0.200 gm.	0.300 gm.	0.400 gm.
	<i>days</i>	<i>gm.</i>			
Oct.	24	46		62	80
"	30	59		75	
Apr.	30	58	64	90	
"	100		135	185	

*Effect of Material Obtained from Alcoholic Extract of Brewers' Yeast*—Three forms of crude vitamin material were used: A, alcoholic extract; B, press-juice; C, precipitate obtained from the latter by means of acetone. Each one of the materials was tested for its capacity to produce normal and accelerated growth.

*A, Alcoholic Extract*—The following figures represent the average increase in weight in 30 days.

Month	Time	Normal increase	Increase in weight in gm.						
			Crude vitamin added per day, gm.						
			0.007	0.010	0.020	0.030	0.040	0.050	0.060
	<i>days</i>	<i>gm.</i>							
Nov.	30	59	0	29	42	65	76	82	96

Thus, it would seem that 0.030 gm. of crude vitamin per day was sufficient to bring about a normal growth and that higher doses accelerated the rate of growth in proportion to the increase in the daily doses of the active material. On 0.060 gm. per day the ratio to normal growth was 1.7:1, which was the same as on 0.400 gm. of the dry yeast.

*B, Press-Juice*—Table III represents the results of the experiments with press-juice.

This material seems more potent than the alcoholic extract, inasmuch as 0.0063 gm. per day sufficed to maintain normal growth. On a daily dose of 0.0175 gm. the ratio-between the treated and normal was 1.8:1, and on a daily dose of 0.125 gm. the ratio was 2.42:1.

TABLE III

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of Press-Juice*

Month	Time	Normal increase	Yeast juice added per day, cc.						
			0.05	0.075	0.1	0.15	0.25	0.5	1.0
			Dry residue added per day, gm.						
			0.0063	0.0088	0.0125	0.0175	0.026	0.0513	0.125
	days	gm.							
Apr.	18	26	26	32	38	52	45	53	63
"	24	35	33	43	47	63			

TABLE IV

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of Acetone Precipitate Obtained from Press-Juice*

Month	Time	Normal increase	Acetone precipitate added per day, gm.						
			0.005	0.0070	0.010	0.015	0.025	0.050	0.100
	days	gm.							
Apr.	18	26				45	40	52	56
"	24	35			48	59			
May	12	22	22	27					

*C, Acetone Precipitate*—Table IV represents the results of the experiments with the acetone precipitate obtained from press-juice.

Comparing the ratios of growth on administration of materials B and C, it is evident that the acetone precipitate retains nearly the total efficiency of the press-juice. Thus, on a daily dose of 0.05 gm. the ratios are 2:1 in both cases.

*Concentration of Crude Vitamin Material by Means of Silica Gel<sup>3</sup>*

*Preparation of Material*—100 gm. of the crude material were triturated in 4 liters of water. Part remained undissolved and was separated from the aqueous part by filtration. To the filtrate 800 gm. of silica gel were added with stirring, and the mixture was maintained at pH 3. After 10 minutes the silica gel was filtered off and washed with very small portions of water. The operation was repeated twice. The silica from the three extractions was combined and suspended in 6 liters of water and to it a solution of lithium hydroxide was added until pH 9.8 was attained. After stirring for 20 minutes, the mixture was filtered, the filtrate rendered neutral by means of a solution of hydriodic acid, and

TABLE V

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of Silica Concentrate (Concentrate 159)*

Month	Time	Normal increase	Concentrate added per day, gm.								
			0.003	0.005	0.007	0.010	0.020	0.030	0.040	0.050	0.060
	days	gm.									
Apr.	12	16	19	22	26	28	33	37	37	43	56
"	18	26		31	32	39	44	53	53	59	73
"	24	35		38	45	51	55	71	71	73	89
"	30	46		47	55	57	63	85	84	88	106

then concentrated to 100 cc. It was allowed to stand in the refrigerator until precipitation of the small amount of silica gel still remaining in suspension was completed. A small portion of alcohol was then added and the mixture filtered. The filtrate was further concentrated and acetone was added to the concentrate until precipitation was completed. The yield of the dried product was about 18 to 20 gm. The dried precipitate was tested for its efficiency, and the results are given in Table V.

Thus, as compared with the crude vitamin preparation, the minimal dose required to bring about normal growth was in the ratio of 3:25. The material was not improved, however, in the same degree with respect to producing a growth above normal. On daily doses of 0.050 gm. the ratio was as 1.4:1.8, and on 0.060

<sup>3</sup> We wish to express our appreciation to the Silica Gel Corporation for their generosity in supplying the silica gel for this work.

gm. as 1.6:2.3. This fact may be explained by assuming that the silica concentrate contained in different proportions the several factors necessary for growth.

In addition, more effective material was prepared through the agency of silica gel when, instead of the alcoholic extract, press-juice was used as starting material, thus showing that the extrac-

TABLE VI

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of the Silica Concentrate Obtained from Press-Juice*

Month	Time	Normal increase	Silica concentrate added per day, gm.	
			0.002	0.005
	days	gm.		
May	12	22	23	37
"	18	30	34	52
"	24	43	45	

TABLE VII

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of the Purified Silica Concentrate (Concentrate 640 Obtained from Press-Juice)*

Month	Time	Normal increase	Concentrate added per day, gm.	
			0.002	0.005
	days	gm.		
May	12	22	22	37
"	18	33	34	51
"	24	44	45	

tion with dilute alcohol is to some extent deleterious to some of the factors contained in the growth-promoting material.

From Table VI it may be seen that daily doses of 0.005 gm. of this concentrate produced a growth higher than normal in the ratio of 1.7:1, while the same dosage of the concentrate prepared from the alcoholic extract was only capable of maintaining normal growth.

*Further Concentration of Material Described Above*—The material prepared by silica absorption was further concentrated in the

following manner. 3.0 gm. of the dry powder were dissolved in 10.0 cc. of water and to this solution were added 100 cc. of ethyl alcohol containing 1 cc. of 70 per cent hydriodic acid. The precipitate thus formed was centrifuged and then washed with alcohol until the washings were free of iodide ions. The precipitate contained the active material, whereas the material obtained from the filtrate was inactive. The results are shown in Table VII.

Thus, when administered alone this material did not possess greater potency than the original material.

Many other ways of concentrating the material absorbed by silica gel were tried, but most of the preparations obtained by the various methods were found to be effective for only relatively short intervals (of 1 or 2 weeks) after which growth proceeded at a very low rate. These failures naturally suggested the possibility of the existence of more than one factor contained in the original growth-promoting material. The existence in the yeast of two factors, one antineuritic and the other growth-promoting, was suggested some time ago by Emmett and Luros,<sup>4</sup> Funk and Dubin,<sup>5</sup> and Levene and Muhlfield.<sup>6</sup> It was, however, Goldberger and his associates<sup>7</sup> and Chick and Roscoe<sup>8</sup> who made the important discovery that one factor (the antineuritic according to Goldberger) was heat-unstable ( $B_1$ ), whereas the second (antipellagra) was heat-stable ( $B_2$ ). These discoveries were made at a time when our own experiments had convinced us that our purified silica concentrates were lacking in some of the factors required for maintenance of normal growth. The findings of Goldberger furnished a method for detecting which of the two factors predominated in the purified silica concentrate. Indeed, it was established that this material consisted in the main of the heat-labile antineuritic fraction. The solution remaining after the silica extraction should furnish the source for the heat-stable factor. This expectation was actually realized.

<sup>4</sup> Emmett, A. D., and Luros, G. O., *J. Biol. Chem.*, **43**, 265 (1920).

<sup>5</sup> Funk, C., and Dubin, H. E., *Proc. Soc. Exp. Biol. and Med.*, **19**, 15 (1921-22).

<sup>6</sup> Levene, P. A., and Muhlfield, M., *J. Biol. Chem.*, **57**, 341 (1923).

<sup>7</sup> Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U.S.P.H.S.*, **41**, 297 (1926).

<sup>8</sup> Chick, H., and Roscoe, M. H., *Biochem. J.*, **21**, 698 (1927). See also Narayanan, B. T., and Drummond, J. C., *Biochem. J.*, **24**, 19 (1930).



*Vitamin B<sub>1</sub> Potency of Material Prepared from Silica Concentrate*—In order to determine the content of factor B<sub>1</sub> in the silica

TABLE VIII

*Average Increase in Weight (in Gm.) of Rats on Administration of Progressively Increased Daily Doses of Fractions B<sub>1</sub> and B<sub>2</sub>*

Fraction B<sub>2</sub> was from Concentrate 447.

Month	Time	Normal increase	B <sub>2</sub> , 0.030 gm. per day (constant); B <sub>1</sub> (Con- centrate 519) added per day (variable)			
			0.0002 gm.	0.0005 gm.		
	<i>days</i>	<i>gm.</i>				
Feb.	12	18	24	33		
"	18	27	32	44		
"	24	36	38	53		
"	30	47	50	62		
			B <sub>1</sub> (Concentrate 159), 0.0002 gm. per day (constant); B <sub>2</sub> added per day (variable)			
			0.040 gm.	0.050 gm.	0.060 gm.	
Feb.	12	18	21	23	26	
"	18	27	30	32	36	
"	24	36	38	42	46	
			B <sub>2</sub> , 0.060 gm. per day (constant); B <sub>1</sub> (Con- centrate 159) added per day (variable)			
			0.003 gm.	0.005 gm.		
Feb.	12	18	23	32		
"	18	27	32	45		
"	24	36	40	54		
			B <sub>1</sub> (Concentrate 519), 0.0001 gm. per day (constant); B <sub>2</sub> added per day (variable)			
			0.020 gm.	0.040 gm.	0.050 gm.	0.060 gm.
Apr.	12	16	14	19	20	22
"	18	26	18	27	30	33
"	24	35	21	34	37	40

concentrate, daily doses of this concentrate were added to crude material which had been heated to 140° in an autoclave, a procedure which destroyed the heat-labile factor B<sub>1</sub>, leaving the factor B<sub>2</sub> intact. In preliminary experiments it was found that 0.030 gm. of this material was a sufficient daily dose of factor B<sub>2</sub>.

Table VIII contains the results of several such experiments, from which it will be seen that when a sufficiently large dose of the heat-stable material was added to the diet, less than 0.0001 gm. of the B<sub>1</sub> concentrate sufficed to maintain normal growth. It may also be seen that with higher daily doses of the B<sub>1</sub> concentrate, a growth rate higher than normal can be attained. However, the minimum dose has to be multiplied many times before a growth rate above normal can be obtained, whereas increasing the dry yeast by about 50 per cent of the minimum requirement results in a 50 per cent increase in the rate of growth. Is this an indication that the sum of the purified silica concentrate and the heated material does not contain all the factors present in the crude material, or does it indicate that the factors B<sub>1</sub> and B<sub>2</sub> are impaired in some of their activities? These questions cannot as yet be answered.

It must be emphasized that the values given for normal growth are the averages of four experiments each. The values given for the concentrated materials (Concentrates 159 and 519) are representative of samples of a number of experiments. Occasionally it was found that the materials obtained in exactly the same manner were less potent than those discussed; as a rule, however, the materials were of the same degree of potency. All samples were, of course, very complex mixtures and all contained in the neighborhood of 25 per cent of mineral matter, including the phosphoric acid bound in ester form (mainly nucleic acid derivatives). The composition of Concentrate 159 was C 34.71, H 5.36, N 6.80, and P 3.00. Calculated as ash-free material, the composition was C 39.85, H 6.15, N 7.80, and P 3.44. Several other samples had approximately the same composition.

At this phase of the work, further purification and concentration of the heat-labile fraction was interrupted and attention was directed to the concentration of the heat-stable fraction.

#### *Concentration of Fraction B<sub>2</sub>*

This material was obtained from the solution remaining after extracting the solution of the crude material with silica gel. Repeating the extraction with silica gel six times leaves a solution which, after neutralization with lithium hydroxide, concentration, and precipitation with acetone, yields a precipitate (Concentrate

546) which is totally inactive when given by itself. Combined with the heat-labile fraction, in doses incapable by themselves of maintaining growth, it leads to normal growth. (See Table IX.)

3.0 gm. of Concentrate 546 were then dissolved in 10 cc. of water and allowed to flow slowly into alcohol containing 0.5 cc. of a 70 per cent hydriodic acid solution. A precipitate formed which was washed with alcohol until the washings no longer contained hydriodic acid (Concentrate 547). (See Table X.)

TABLE IX

*Average Increase in Weight (in Gm.) of Rats on Administration of Purified Fractions B<sub>1</sub> and B<sub>2</sub>*

Fraction B<sub>1</sub>, 0.0002 gm. of Concentrate 519 per day (constant).

Fraction B<sub>2</sub>, Concentrate 546 in increasing doses per day (variable).

Month	Time	Normal increase	B <sub>2</sub> added per day, gm.	
			0.005	0.007
	<i>days</i>	<i>gm.</i>		
May	12	22	22	24
"	18	34	32	35

TABLE X

*Average Increase in Weight (in Gm.) of Rats on Administration of Purified Fraction B<sub>1</sub> and Further Purified Fraction B<sub>2</sub>*

Fraction B<sub>1</sub>, 0.0002 gm. per day (constant).

Fraction B<sub>2</sub>, Concentrate 547 in increasing doses in gm. per day (variable).

Month	Time	Normal increase	B <sub>2</sub> added per day, gm.				
			0.0005	0.001	0.002	0.003	0.005
	<i>days</i>	<i>gm.</i>					
June	12	20	19.0	24	25	30	37

Each value is the average of several experiments. At this stage the work was discontinued. It is now being published in the belief that the materials of the type of Concentrates 519 and 547 may serve as starting material for further concentration of the growth-promoting principle and also in the belief that the materials may be useful in physiological studies when it is found desirable to use the growth-promoting material in a concentrated form.

## GLUCOSIDE FORMATION IN THE COMMONER MONOSES

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The present communication deals with the rates of formation of the furanosides and pyranosides of the commoner monoses, as a function of their configuration. The original objective of the investigation was of a purely practical nature; namely, the desire to establish the optimal conditions for the preparation of certain furanosides. It is generally known that furanosides are formed with greater velocity than the corresponding pyranosides and methods of preparation have been based on this difference. Moreover, although it is well known that the configuration of the sugar affects the rates of glucoside formation, no comparative information of a quantitative nature has been hitherto available. Studies have now been made on several monoses as regards their furanose and pyranose formation under comparable conditions.

The data are presented in Table I and in Figs. 1 and 2. Fig. 1 is a set of graphs showing the changes in the proportions of free sugar and of furanose and pyranose. It will be noted that for each sugar investigated, the amount of furanose rises to a maximum and then gradually decreases. Particular interest arises from the fact that this maximum production is not always clearly indicated by the curves (Fig. 2) of optical rotation (for example, mannose, fructose, arabinose, xylose) and yet it is at this point that the experiment should be interrupted in order to obtain the maximum yield of furanose. In the cases of ribose and xylose, in which we were particularly interested, the proportion of furanose approaches 100 per cent so that modification of the present experimental conditions was unnecessary. However, in the case of other sugars, it is possible that the proportion of furanose could be appreciably increased by changes in temperature, or in

TABLE I

*Glucoside Formation at 25° from Reduction Determinations*

Sugar	Time	Co. 0.01 N thiosulfate		Mg.		Free sugar, per cent		Corrected, per cent*			Distribution of sugar, per cent		
		Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	A	B	B <sub>c</sub>	Free	Glucoside	
												γ	Normal
Glucose	hrs.												
	0	9.47	9.84	3.17	8.86	102.4	95.5	101	98	98	101	-3	2
	1	7.07	9.68	2.38	8.72	76.8	93.8	76	96	96	76	20	4
	3	4.48	9.58	1.52	8.63	49.0	92.9	48	95	95	48	47	5
	7	2.41	9.30	0.82	8.38	26.4	90.1	26	92	92	26	66	8
	24	1.08	8.18	0.37	7.36	11.9	79.3	12	81	80	12	68	20
Present	48	0.95	7.46	0.32	6.72	10.3	72.2	10	74	73	10	63	27
				3.10	9.30								
Mannose	0	9.56	9.52	3.25	8.57	104.8	92.2	99	94	94	99	-5	6
	1	8.32	9.04	2.83	8.14	91.3	87.5	86	89	88	86	2	12
	3	6.29	8.70	2.14	7.83	69.0	84.2	65	86	85	65	20	15
	7	3.81	7.79	1.30	7.02	41.9	75.5	40	77	76	40	36	24
	24	0.32	3.62	0.11	3.26	3.6	35.0	3	36	33	3	30	67
	48	0.10	1.38	0.03	1.24	1.0	13.3	1	14	9	1	8	91
Present				3.10	9.30								
Galactose	0	7.22	7.62	3.14	3.32	101.4	107.1	101	107	108	101	7	-8
	1	5.45	6.98	2.38	3.05	76.9	98.4	75	98	98	75	23	2
	3	3.28	6.15	1.45	2.68	46.8	86.5	46	86	84	46	38	16
	7	1.45	5.07	0.66	2.22	21.3	71.6	21	72	69	21	48	31
	24	0.42	3.63	0.19	1.60	6.1	51.6	6	52	46	6	40	54
	48	0.09	2.70	0.04	1.20	1.3	38.7	1	39	31	1	30	69
Present				3.10	3.10								
Fructose	0	6.30	11.19	2.00	3.58	64.5	115.5	67	101	101	67	34	-1
	1	0.40	11.42	0.13	3.66	4.2	118.1	4	104	104	4	100	-4
	3	0.33	11.39	0.11	3.65	3.5	117.7	4	103	103	4	99	-3
	7	0.29	11.19	0.09	3.58	2.9	115.5	3	101	101	3	98	-1
	24	0.19	10.56	0.06	3.37	1.9	108.7	2	95	95	2	93	5
	48	0.30	10.64	0.10	3.40	3.2	109.6	3	96	96	3	93	4
Present	216	0.27	7.47	0.09	2.54	2.9	81.9	3	72	71	3	68	28
				3.10	3.10								

\* The letters in this column refer to the percentages defined in the text; A, before hydrolysis, B, after hydrolysis, B<sub>c</sub>, after hydrolysis corrected.

TABLE I—Concluded

Sugar	Time	Co. 0.01 N thiosulfate		Mg.		Free sugar, per cent		Corrected, per cent*			Distribution of sugar, per cent		
		Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	A	B	B <sub>c</sub>	Free	Glucoside	
												γ	Normal
Rhamnose	hrs.												
	0	9.66	9.51	3.14	8.66	100.3	92.3	105	96	96	105	-9	4
	1	8.36	9.12	2.65	8.30	84.7	88.4	89	92	91	89	2	9
	3	7.88	8.64	2.46	7.87	78.5	83.8	83	87	85	83	3	14
	7	6.08	7.57	1.89	6.89	60.4	73.4	64	76	73	64	9	27
	24	2.11	3.91	0.65	3.56	20.8	37.9	22	39	32	22	10	68
Present	48	0.43	1.72	0.13	1.57	4.2	16.7	4	17	8	4	4	92
				3.13	9.39								
Arabinose	0	6.23	9.56	2.25	7.17	87.2	92.7	89	98	97	89	8	3
	1	3.38	8.49	1.24	6.37	48.1	82.3	49	87	82	49	33	18
	3	0.81	7.34	0.29	5.51	11.2	71.2	11	75	69	11	58	31
	7	0.13	3.75	0.05	2.81	1.9	36.3	2	38	16	2	14	84
	24	0.09	2.99	0.03	2.24	1.2	28.9	1	30	5	1	4	95
	48	0.09	2.66	0.03	2.00	1.2	25.8	1	27	1	1	0	99
Present				2.58	7.74								
Lyxose	0	7.66	9.55	2.34	7.16	90.7	92.6	91	96	96	91	-5	4
	1	4.52	8.34	1.57	6.26	60.8	80.9	61	83	81	61	20	19
	3	1.63	7.11	0.57	5.33	22.1	68.9	22	71	68	22	46	32
	7	0.32	5.43	0.11	4.07	4.3	52.6	4	54	49	4	45	51
	24	0.14	2.17	0.05	1.63	1.9	21.1	2	22	13	2	11	87
	48	0.11	1.05	0.04	0.79	1.6	10.2	2	11	1	2	-1	99
Present				2.58	7.74								
Ribose	0	6.35	9.75	2.47	7.31	95.8	94.5	93	97	96	93	3	4
	1	0.08	9.68	0.03	7.26	1.2	93.9	1	96	94	1	93	6
	3	0.08	9.73	0.03	7.30	1.2	94.3	1	96	94	1	93	6
	7	0.09	9.32	0.04	6.99	1.6	90.3	2	92	88	2	86	12
	24	0.09	8.84	0.04	6.63	1.6	85.6	2	87	81	2	79	19
	48	0.12	7.73	0.05	5.80	1.9	74.9	2	76	65	2	63	35
Present				2.58	7.74								
Xylose	0	7.50	9.64	2.49	7.23	96.6	93.3	98	95	94	98	-4	6
	1	2.00	9.70	0.64	7.28	24.8	94.1	25	96	95	25	70	5
	3	0.43	9.51	0.14	7.13	5.4	92.1	5	94	93	5	88	7
	7	0.36	9.29	0.12	6.97	4.6	90.1	5	92	91	5	86	9
	24	0.24	7.78	0.08	5.84	3.1	75.3	3	78	74	3	71	26
	48	0.27	6.68	0.09	5.01	3.5	64.6	3	66	61	3	58	39
Present				2.58	7.74								

concentration of sugar or catalyst. The relative furanoside rates for the different sugars may be obtained from the slopes of the curves at zero time since the whole of the sugar is then available for furanoside (and pyranoside) formation. Arranged in the order

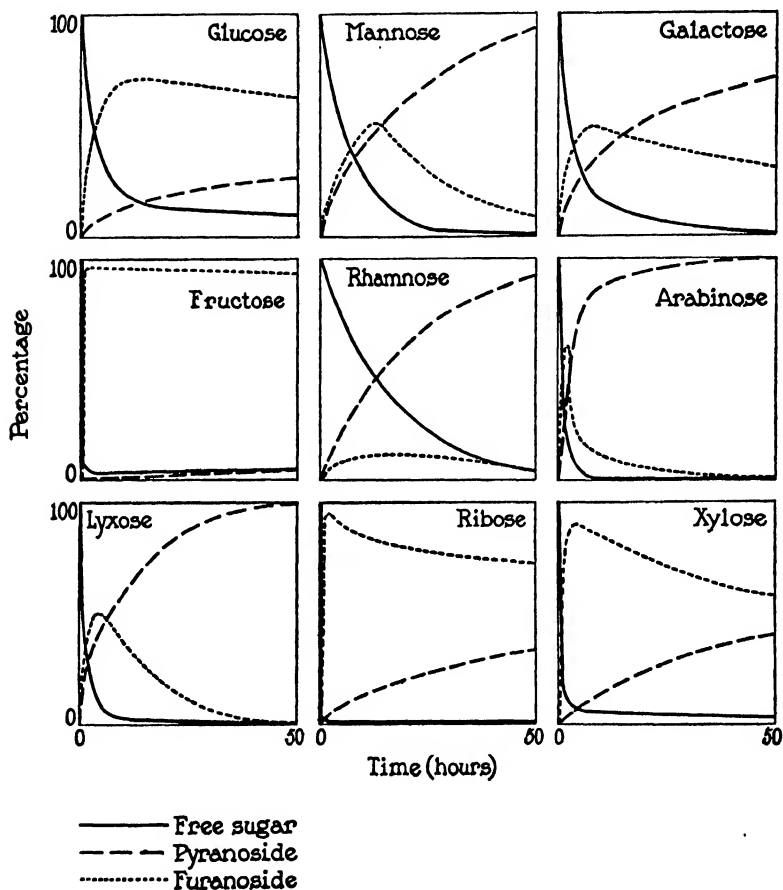


FIG. 1. Percentages of free sugar, furanoside, and pyranoside during glucoside formation at 25° in methyl alcohol containing 0.5 per cent hydrogen chloride.

of decreasing velocities of furanoside formation the sequence is fructose — ribose and xylose — galactose, glucose, and lyxose — mannose — rhamnose.

The curve of pyranoside formation offers difficulties of interpretation for the reason that the pyranosides are formed not only from the reducing sugar, but also by transformation of the furanosides. Consequently, the rate of formation is affected both directly by the configuration of the sugar and indirectly by the effect of configuration on the formation and transformation of the furanosides. The direct effect may be obtained from the slopes at zero time of the pyranoside curves (Fig. 1), and the sugars may thus be arranged in order of decreasing pyranoside rate in the series: arabinose — lyxose — mannose and rhamnose — galactose — xylose — glucose and ribose — fructose. The indirect formation is particularly noticeable in the cases of ribose and xylose. In these sugars the rate of pyranoside formation may be determined entirely by the rates of transformation of the furanosides inasmuch as after a very short interval almost no free sugar remains and yet pyranoside formation continues to take place.

*Method of Determining the Proportion of Furanosides and Pyranosides*—For purposes of estimation, furanosides and pyranosides may be differentiated by two properties, first, their optical rotation, and second, their rates of hydrolysis with dilute mineral acids. The optical method can be applied with accuracy only in a system of two components, for in a system of three or more components a knowledge of the specific rotations is not sufficient for the determination of the proportions of each. On the other hand, the character of the rotation curve may, in some cases, indicate the formation of only one or of more than one ring type of glucoside. In other instances, the curves fail to reveal the formation of two glucosides even when such formation is demonstrable by other methods. For these reasons the method of hydrolysis was resorted to. The principle of the method is very simple; namely, mild conditions suffice for the hydrolysis of furanosides whereas more drastic treatment is required for that of pyranosides.

If conditions could be found which would leave the pyranoside intact and yet would lead to complete hydrolysis of the furanoside, then  $A$ , the percentage of reducing sugar before such hydrolysis, and  $B$ , the percentage of reducing sugar after hydrolysis, are the only data which would be required.  $A$ , then, is the percentage of unreacted sugar at the particular moment,  $B - A$  is the corresponding percentage of sugar present as furanoside, and  $100 - B$



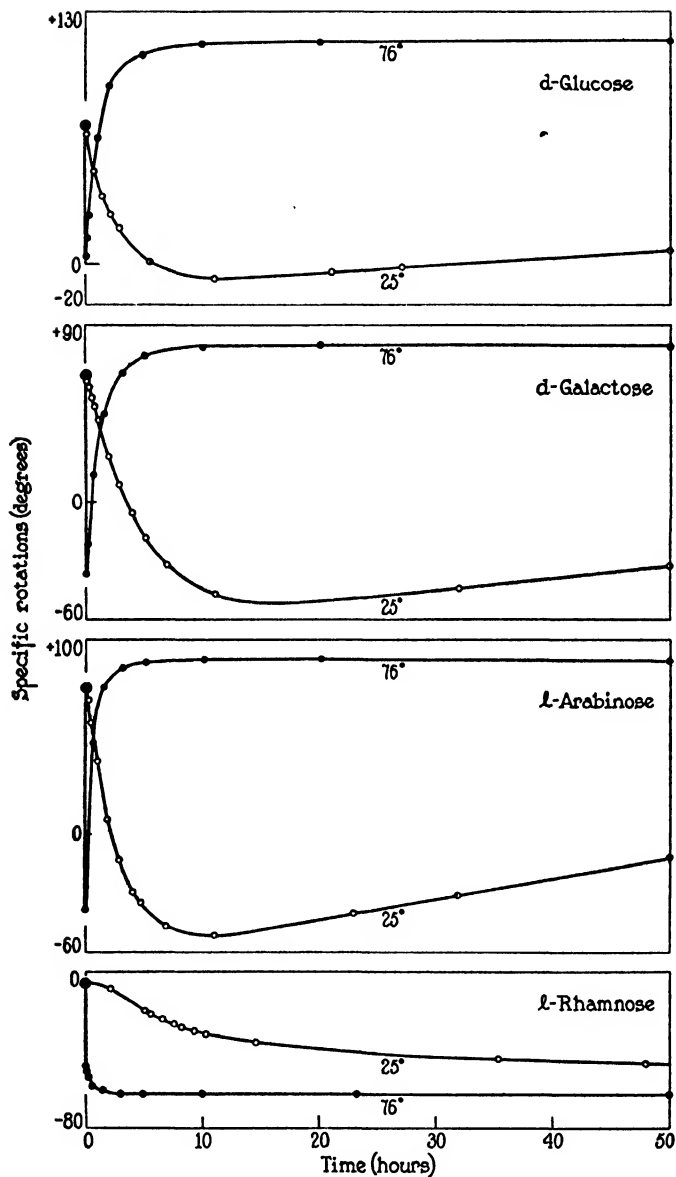


FIG. 2, a

FIGS. 2, a AND 2, b. Specific rotations during glucoside formation in methyl alcohol containing 0.5 per cent hydrogen chloride.

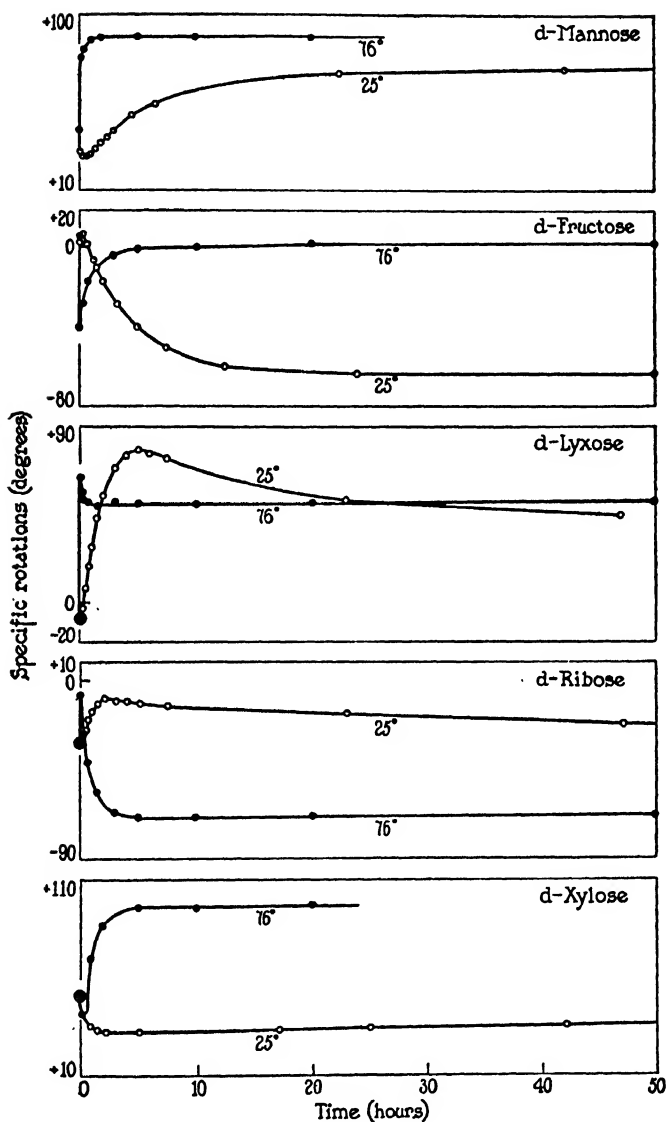


FIG. 2, b

is the percentage of sugar present as pyranoside. Actually, however, the hydrolysis of the furanoside is probably not invari-

ably quantitative nor is the hydrolysis of the pyranoside always negligible. A corrected value for  $B$  may be calculated by using the formula,  $B_c = \frac{100 (B - P)}{100 - P}$ , which is derived on the assumption that  $P$ , the percentage hydrolysis of the pyranoside, is constant throughout the experiment, or in other words, that the amount of such hydrolysis in any sample is proportional only to the amount of pyranoside present in that sample. In actual fact  $P$  was determined by hydrolyzing samples which had reached optical equilibrium and hence probable equilibrium of  $\alpha$  and  $\beta$  forms of the pyranoside. The proportion of  $\alpha$  to  $\beta$  does not, however, remain constant during the experiment so that even the corrected values  $B_c$  are still generally too large. However, this error is at least in part compensated by the incomplete hydrolysis of the furanoside mentioned above. *It must be emphasized that the values obtained from the hydrolysis data are a satisfactory first approximation, permitting the determination of the time of maximum furanoside formation, and illustrating the differences in the rates of formation of furanosides and pyranosides as a function of the configuration of the sugar.*

In connection with the corrections which were used in the work, it may be mentioned that small deviations from the accepted values were found for the reducing power of our samples of sugars, particularly after they had been subjected to treatment with acid under conditions used for glucoside hydrolysis. For this reason the reducing value was determined for each sugar, both before and after acid treatment, and the factors thus obtained were used in making the calculations.

## EXPERIMENTAL

### *Materials*

With the exception of the glucose, which was an anhydrous, reagent grade product, all of the sugars used were prepared in this laboratory and were purified by crystallization. They were dried over phosphorus pentoxide to almost constant weight; the moisture was then determined (the maximum was 0.3 per cent) and the corresponding correction was applied to the weight of the samples.

### *Procedure*

The calculated quantity of the dried sugar was weighed into a volumetric flask and dissolved in anhydrous methyl alcohol (reagent grade, acetone-free). The calculated volume of a standardized solution of dry hydrogen chloride in anhydrous methyl alcohol was added with a pipette, the mixture was immediately diluted to the desired volume with more anhydrous methyl alcohol and thoroughly mixed by shaking. The hydrogen chloride concentration in the glucoside mixtures was 0.5 per cent and the sugars were 0.344 molal. Experiments were made at room temperature and at 76°. In the latter case, portions of the solution were sealed in Pyrex test-tubes which were heated in a bath of boiling carbon tetrachloride for the required intervals of time, then removed and cooled immediately in ice.

### *Analytical*

Samples of 5.0 cc. were removed as required, placed in a volumetric flask, and 2 cc. of a 0.4 N sodium carbonate solution (17 per cent excess over that required for the hydrochloric acid present) were added. After shaking well, the mixture was diluted almost to volume, cooled to room temperature, diluted to 10.0 cc., and well mixed. These samples were used for analysis.

For the determination of the free sugar the neutralized samples were analyzed without treatment as it was found that the small amount of methyl alcohol present had no effect on the reduction determinations. However, as it was learned from preliminary experiments that the hydrolysis of the furanosides was materially decreased in the presence of methyl alcohol, its removal prior to hydrolysis was necessary. The samples to be hydrolyzed were therefore placed in test-tubes fitted with distilling heads and the solutions were concentrated almost to dryness under diminished pressure. 3 to 4 cc. of water were then added and the evaporation was repeated. The residue was diluted to about  $3\frac{1}{2}$  cc. with water and 0.5 cc. of 0.4 N hydrochloric acid<sup>1</sup> was added. The tubes were stoppered with small funnels, heated in the steam bath for 10

<sup>1</sup> For certain sugars 0.2 N hydrochloric acid was used, and the heating was 5 minutes in some cases. The times and concentrations are listed in Tables IV and V.

minutes,<sup>1</sup> and then removed and cooled. The solutions were neutralized with the theoretical equivalent of 0.4 N sodium carbonate or sodium hydroxide, diluted to 5 cc., and the analyses were then performed as for the unhydrolyzed samples.

Two analytical methods were employed, the Hanes<sup>2</sup> modification of the Hagedorn-Jensen<sup>3</sup> method, and a micro modification of the Willstätter<sup>4</sup> hypiodite method. For the former procedure 1.0 cc. portions of the neutralized samples were used and for the latter 3.0 cc. portions.

For the micro hypiodite analyses (Procedure A) the samples were placed in large Pyrex tubes and diluted to  $7\frac{1}{2}$  cc. with distilled water. 1.5 cc. of 0.1 N iodine-potassium iodide solution were added and then, drop by drop, 1 cc. of 0.3 N sodium hydroxide. After standing 15 minutes at room temperature there were added 2 cc. of 0.2 N potassium iodide and 0.2 cc. of 5 N sulfuric acid, and the free iodine was titrated with N/70 sodium thiosulfate solution with starch as an indicator.

It was found that this procedure gave results which were considerably less than the theoretical in the case of those sugars which have *cis* hydroxyls on carbon atoms 2 and 3 (*i.e.*, mannose, rhamnose, lyxose, and ribose) so that for these sugars the procedure was somewhat modified. In Procedure B, 3.0 cc. of sample were diluted with water to  $7\frac{1}{2}$  cc. volume as before. After addition of 1.5 cc. of 0.1 N iodine-potassium iodide the tubes were placed in ice and allowed to cool for several minutes, and were kept in this ice bath except during the additions of alkali. The 0.3 N sodium hydroxide was added in small portions at 2 minute intervals, the tubes being well shaken during each addition. The amounts added were 0.2, 0.2, 0.1, 0.1, 0.1, 0.1, and 0.1 cc., a total of 0.9 cc. requiring 12 minutes for complete addition. The tubes were left in the ice bath for an additional 3 minutes and were then transferred to a beaker of water at room temperature, in which they were left for a further 15 minutes. Potassium iodide and sulfuric acid were added as before and the titration was performed similarly. By means of this modification in technique, the values found for

<sup>1</sup> Hanes, C. S., *Biochem. J.*, **23**, 99 (1929).

<sup>2</sup> Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923).

<sup>4</sup> Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, **51**, 780 (1918).

these abnormal sugars were increased from an average of 74.8 per cent of the theoretical to an average of 97.4 per cent.

### *Hydrolysis of Normal Glucosides*

Samples of the sugar, methyl alcohol, and hydrogen chloride solutions were heated at 76° in sealed tubes until no further change in rotation occurred, and the amount of free sugar was then de-

TABLE II  
*Stability of Normal Glucosides*

Sugar	Glucoside heating hrs.	Cc. 0.01 N thiosulfate		Sugar, mg.		Sugar present, mg.	Free sugar, per cent		Hydrolysis with HCl at 100°
		Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis		Before hydrolysis	After hydrolysis	
Glucose	15	0.06	0.07	0.02	0.16	3.10	1	5	0.1 N, 10 min
Mannose	4.5	0.06	0.47	0.02	0.16	3.10	1	5	0.1 " 10 "
Galactose	15	0.08	1.43	0.04	0.65	3.10	1	21	0.1 " 10 "
			0.73		0.34			11	0.05 " 10 "
Fructose	10	0.61	0.40	0.20	0.13	3.10	6	4	0.1 " 10 "
Rhamnose	4	0.05	1.04	0.02	0.32	3.13	1	10	0.1 " 10 "
Arabinose	10	0.14	2.24	0.05	0.82	2.58	2	32	0.1 " 10 "
			1.82		0.66			26	0.05 " 5 "
Lyxose	7	0.08	1.89	0.03	0.72	2.58	1	28	0.1 " 10 "
			0.67		0.25			10	0.05 " 5 "
Ribose	6	0.18	2.87	0.07	1.12	2.58	3	43	0.1 " 10 "
			2.04		0.80			31	0.05 " 5 "
Xylose	12	0.11	0.96	0.03	0.31	2.58	1	12	0.1 " 10 "

terminated by analysis. The amount was invariably found to be very small. Portions were freed of methyl alcohol by concentration as described above and were then hydrolyzed with hydrochloric acid of various strengths and for different periods of time. Some of the data thus obtained are given in Table II. These factors were used in calculating the corrected reducing values according to the formula on p. 188.

*Analytical Factors*

The data of Sobotka and Reiner<sup>5</sup> were used as standards for the Hanes modification of the Hagedorn-Jensen method but as they did not include lyxose<sup>6</sup> a standard solution of this sugar was prepared and the factors were determined. The results are given in Table III.

The samples of sugars used were all subjected to analysis, both before and after acid treatment, in order to obtain their exact reducing equivalent. The reducing values found by the Hagedorn-Jensen (Hanes) method are given in Table IV and those found by the hypiodite method in Table V.<sup>7</sup> Before acid treatment the deviations from the theory were small but after treatment the deviations with the Hagedorn-Jensen method became

TABLE III  
*Reducing Values by Hagedorn-Jensen (Hanes) Method. Cc. 0.01 N  
Thiosulfate*

Sugar, mg.....	1.0	2.0	3.0	4.0
Lyxose.....	2.83	5.81	8.73	
Cellobiose.....	2.58	5.10	7.61	10.09

quite large as will be seen from Table IV. It was for this reason that the hypiodite oxidation was employed in the majority of cases for the samples after hydrolysis.

*Optical Rotation*

The rotations were measured on the 76° samples (previously cooled in ice) by warming them rapidly to room temperature and reading them in a 2 dm. tube with sodium D light. In the experiments conducted at room temperature samples were placed in

<sup>5</sup> Sobotka, H., and Reiner, M., *Biochem. J.*, **24**, 394 (1930).

<sup>6</sup> Cellobiose standards were also made but this sugar was not used for glucoside experiments. The data are given, however, for possible future reference.

<sup>7</sup> Cellobiose, lactose, and maltose (the last probably not quite pure) are appended for possible reference although they were not actually used in the glucoside formation experiments.

TABLE IV  
Correction Factors for Hagedorn-Jensen (Hanes) Method

Sugar	Present, mg.	Cc. 0.01 N thiosulfate		Sugar found, mg.		Free sugar, per cent		Hydrolysis with HCl at 100°
		Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	
Glucose.....	3.0	9.06	10.54	3.03	3.52	101	117	0.1 N, 10 min.
Mannose.....	3.0	9.34	8.44	3.18	2.87	106	96	0.1 " 10 "
Galactose.....	3.0	7.03	6.86	3.06	2.99	102	100	0.1 " 10 "
Fructose.....	3.0	9.06	10.69	2.88	3.42	96	114	0.1 " 10 "
Rhamnose....	3.0	8.96	8.00	2.86	2.52	95	84	0.1 " 10 "
Arabinose....	3.0	8.22	8.74	2.93	3.11	98	104	0.05 " 5 "
Lyxose.....	3.0		9.36		3.20	100	107	0.05 " 5 "
Ribose.....	3.0	8.00	8.46	3.09	3.26	103	109	0.05 " 5 "
Xylose.....	3.0	8.94	9.92	2.97	3.29	99	110	0.1 " 10 "
Cellobiose....	3.0		8.03		3.16	100	105	0.05 " 10 "
Lactose.....	3.0	6.60	6.75	2.88	2.95	96	98	0.05 " 5 "
Maltose.....	3.0	6.36	6.64	2.62	2.73	87	91	0.05 " 5 "

TABLE V  
Correction Factors for Micro Hypiodite (Willstätter) Method

Sugar	Present, mg.	Cc. 0.01 N thiosulfate		Sugar found, mg.		Free sugar, per cent		Hydrolysis with HCl at 100°
		Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	
Glucose.....	5.0	5.40	5.45	4.86	4.91	97.2	98.2	0.1 N, 10 min.
Mannose*.....	5.0	5.36	5.45	4.83	4.91	96.6	98.2	0.1 " 10 "
Galactose.....	5.0	5.01	5.10	4.51	4.59	90.2	91.8	0.1 " 10 "
Rhamnose*....	5.0	5.19	5.30	4.72	4.82	94.4	96.4	0.1 " 10 "
Arabinose.....	5.0	6.22	6.30	4.67	4.73	93.4	94.6	0.05 " 5 "
Lyxose*.....	5.0	6.52	6.45	4.89	4.84	97.8	96.8	0.05 " 5 "
Ribose*.....	5.0	6.57	6.56	4.93	4.92	98.6	98.4	0.05 " 5 "
Xylose.....	5.0	6.47	6.53	4.85	4.90	97.0	98.0	0.1 " 10 "
Cellobiose....	5.0	2.83	2.93	4.84	5.02	96.8	100.4	0.05 " 10 "
Lactose.....	5.0	2.82	2.99	4.83	5.12	96.6	102.6	0.05 " 5 "
Maltose.....	5.0	2.59	2.78	4.43	4.76	88.6	93.2	0.05 " 5 "

\* Procedure B (see text) was used for these sugars and Procedure A for the others.



glass-stoppered 4 dm. tubes and readings were made at intervals without removing the samples from the tubes. For the longer intervals, new samples were taken from the glass-stoppered volumetric flasks containing the original mixture which was kept at the same temperature as the tubes. The optical data are plotted in Fig. 2.

In order to make certain that the initial readings were independent of the form of the sugar used (whether  $\alpha$  or  $\beta$ ) the rate of

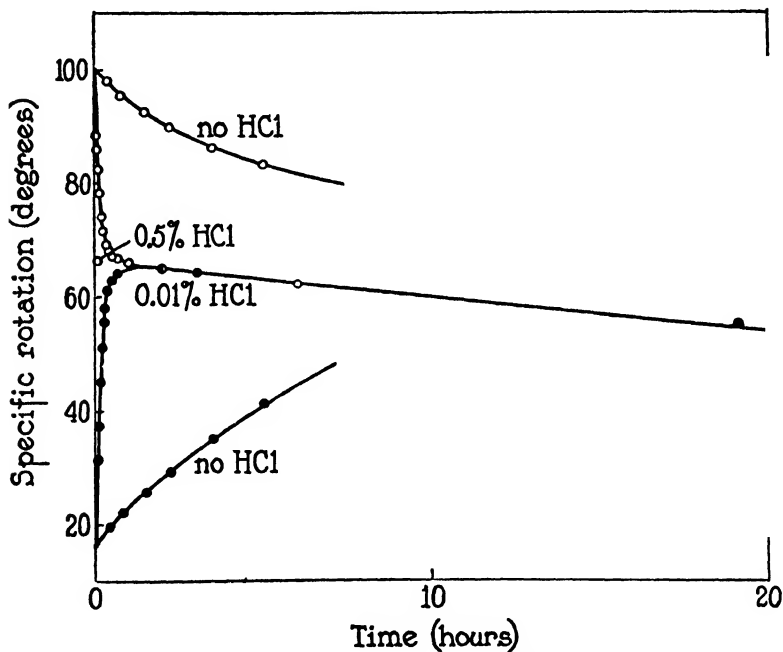


FIG. 3. Mutarotation of  $\alpha$ - and  $\beta$ -glucose at 25° in methyl alcohol

mutarotation of the two forms of glucose in methyl alcohol was determined and the effect of varying amounts of hydrogen chloride was studied. It may be seen from Fig. 3 that the mutarotation is so rapid in the presence of even 0.01 per cent hydrogen chloride that in the presence of 0.5 per cent, the mutarotation would be almost instantaneous and the first reading would represent the equilibrium mixture of the free sugar independently of whether the  $\alpha$  or  $\beta$  form is employed.

## SUMMARY

The glucoside formation of all the commoner monoses has been studied under comparable conditions.

For each sugar the proportion of furanoside rises to a maximum and then decreases. The time required to reach this maximum is in general different for each individual sugar.

The specific rates of furanoside and pyranoside formation are different for each sugar. The ratio of the two rates is likewise in general different for each sugar.



## THE RIBOSEPHOSPHORIC ACID FROM XANTHYLIC ACID

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(Received for publication, January 28, 1932)

The arrangement of the component parts of the nucleotides derived from ribopolynucleotides is formulated on the basis of the assumed analogy of their structure with that of inosinic acid. The latter nucleotide is the only one from which it had been possible to obtain ribosephosphoric acid by acid hydrolysis and the nucleoside by alkaline hydrolysis. From the other nucleotides only the nucleosides had been isolated; all efforts to obtain a ribosephosphoric acid from them were unsuccessful. The failure was attributed to the greater instability of the ribosephosphoric acid entering in the structure of these nucleotides. The instability, in its turn, was explained by assigning to the phosphoric acid group a position different from that in inosinic acid. The arguments in favor of this assumption have been discussed before and need not be repeated here.<sup>1</sup>

In a recent paper Levene and Dmochowski<sup>2</sup> reported on the fact that xanthylic acid, derived from guanylic acid, on standing in aqueous solution at its own pH of 1.9 was partly hydrolyzed with the formation of ribosephosphoric acid. The present communication contains a report on the isolation and properties of this acid, and it may now be stated that the part of the theory of the structure of nucleotides which deals with the arrangement of its components is definitely established.

<sup>1</sup> Levene, P. A., and Yamagawa, M., *J. Biol. Chem.*, **43**, 323 (1920). Embden, G., and Schmidt, G., *Z. physiol. Chem.*, **181**, 130 (1929). Schmidt, G., *Klin. Woch.*, **10**, 165 (1931). Levene, P. A., and Weber, I., *J. Biol. Chem.*, **60**, 707 (1924).

<sup>2</sup> Levene, P. A., and Dmochowski, A., *J. Biol. Chem.*, **93**, 563 (1931).

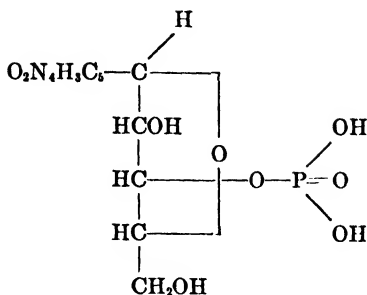
The properties of the ribosephosphoric acid now isolated could be compared with those of the one derived from inosinic acid. The acids were found to differ in several respects. First, they showed different rates of hydrolysis of their phosphoric acid groups, the reaction proceeding twice as fast in the case of the new ribosephosphoric acid. Incidentally, it should be stated that the rates of hydrolysis were compared not directly on the ribosephosphoric acids but on the phosphoribonic acids derived from them (see Table II). This procedure was resorted to for the reason that the phosphoribonic acids were more readily obtained in pure state than were the parent ribosephosphoric acids. Second, differences were observed in the optical changes occurring in course of lactone formation. The changes in rotation observed on the acid derived from inosinic acid were of the same character as those previously reported by Levene and Jacobs<sup>3</sup> and by Levene and Mori,<sup>4</sup> whereas the rotation of the acid obtained from xanthylic acid remained constant during an equal period of time. Inasmuch as in the ribosephosphoric acid from inosinic acid the inorganic component is attached to the ribose in position (5), it was natural to exclude this position in the case of the ribosephosphoric acid derived from xanthylic acid. This assumption is substantiated by the fact that this ribosephosphoric acid forms two glycosides, a furanoside and a pyranoside.

All these facts permit of a more accurate formulation of the structure of xanthylic and hence of guanylic acids. It is now definitely proved that they consist of a ribosephosphoric acid linked glycosidically to the base. Furthermore, the position of the phosphoric acid on the ribose is now restricted to carbon atoms (2) and (3) in view of the facts that position (5) is free and position (4) is engaged in the ring structure, inasmuch as the nucleosides possess the structure of furanosides.<sup>5</sup> Hence, the structure of xanthylic acid may be represented by the formula:

<sup>3</sup> Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **44**, 746 (1911).

<sup>4</sup> Levene, P. A., and Mori, T., *J. Biol. Chem.*, **81**, 215 (1929).

<sup>5</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **94**, 809 (1931-32).



As has already been stated, the phosphoric acid group may with equal justification be allocated to carbon atom (2). This question is now under investigation.<sup>6</sup>

#### EXPERIMENTAL

*Hydrolysis of Guanylic and Xanthylic Acids*—The results of Levene and Dmochowski<sup>2</sup> on the hydrolysis of guanylic and xanthylic acids were confirmed. The estimation of free sugar was performed by the method of Hagedorn and Jensen<sup>7</sup> as modified by Hanes.<sup>8</sup> The phosphorus was determined colorimetrically by the method of Kuttner and Cohn<sup>9</sup> as modified by Raymond and Levene.<sup>10</sup>

In addition, it was found that even at pH 1.5 the rate of hydrolysis of guanylic acid was still very much less than that of xanthylic acid. Also changing the pH of xanthylic acid from 1.9 to 1.5 did not greatly affect its rate of hydrolysis. Therefore, xanthylic acid in an aqueous solution was allowed to stand at its own pH of 1.9, at a constant temperature of 50°, for 3 or 4 days.

*Preparation of Xanthylic Acid*—Xanthylic acid was prepared essentially by the procedure of Levene and Dmochowski.<sup>2</sup> It was

<sup>6</sup> In the monograph "Nucleic acids" by Levene and Bass (New York (1931)) there is an error in Fig. VI on p. 190. The phosphoric acid radicle is given there on position (4) instead of position (2) or (3). It is evident from the formula given there that the hydroxyl of carbon atom (4) is engaged in the ring structure.

<sup>7</sup> Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923).

<sup>8</sup> Hanes, C. S., *Biochem. J.*, **23**, 99 (1929).

<sup>9</sup> Kuttner, T., and Cohn, H. R., *J. Biol. Chem.*, **75**, 517 (1927).

<sup>10</sup> Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **79**, 621 (1928).

found that one-third the amount of potassium nitrite used by them was sufficient and possibly better because the excess nitrite seemed to interfere with the precipitation of lead xanthylate. The xanthylic acid was precipitated from a concentrated aqueous solution by the addition of absolute alcohol. The analysis of this product showed that it was slightly purer than the product obtained by allowing the concentrated aqueous solution to stand in the refrigerator. The xanthylic acid was not completely precipitated by alcohol but for the preparation of ribosephosphoric acid it was not necessary to isolate it from aqueous solution.

Moist sodium guanylate was used in the preparation of xanthylic acid. Therefore, the estimation of yields is not very accurate. Approximately 45 to 50 per cent of the theoretical amount of xanthylic acid was obtained.

*Preparation of Barium Ribosephosphate from Xanthylic Acid*—1 liter of an aqueous solution containing 10 gm. of xanthylic acid was allowed to stand in a constant temperature oven at 50° for 3 or 4 days. The solution was then cooled to 20° with tap water, filtered to free it of precipitated base, and treated with a slight excess of a 10 per cent solution of mercuric sulfate. The precipitate, which was sometimes purple in color, was filtered through a Buchner funnel. The solution was neutralized with barium carbonate and saturated with hydrogen sulfide. The excess gas was removed by aeration and the solution treated with barium hydroxide until it was faintly alkaline to phenolphthalein.

This solution was filtered and then concentrated to about 20 cc. under reduced pressure in a bath at about 40°. If any precipitate of barium carbonate separated during distillation, it was removed by filtration. The resulting solution was treated with an equal volume of absolute alcohol, which precipitated the barium ribosephosphate. This was centrifuged, washed twice with absolute alcohol, and then with ether until it became powdery, and was finally dried in a vacuum desiccator over phosphorus pentoxide. The principal impurities were barium phosphate and barium carbonate. (The nitrogen compounds had been completely removed by the treatment with mercuric sulfate.) The yield was 6 gm. or 60 per cent of the theoretical.

*Direct Preparation of Barium Ribosephosphate from Sodium Guanylate*—Approximately 208 gm. of sodium guanylate (from

1600 gm. of yeast nucleic acid) were converted into xanthylic acid, which was hydrolyzed without isolation. The product was worked up as described above. The yield of barium ribosephosphate was 92 gm. (0.252 mol) or about 49.3 per cent of the sodium guanylate. It had the following composition.

4.108 mg. substance: 21.940 mg. ammonium phosphomolybdate  
 0.1000 gm. " : 0.0670 gm. BaSO<sub>4</sub>  
 C<sub>5</sub>H<sub>8</sub>O<sub>8</sub>PBa. Calculated. P 8.48, Ba 37.59  
 Found. " 7.75, " 39.43

*Purification of Barium Ribosephosphate*—Barium ribosephosphate could not be prepared free from nitrogen compounds unless it first underwent the treatment with mercury sulfate. The resulting barium ribosephosphate was purified by two different methods.

*Method I*—The crude product was dissolved in a small amount of water, diluted to 2 or 3 times its volume, and the precipitated barium carbonate and phosphate removed by centrifuging the solution. The clear solution was then reprecipitated by pouring into absolute alcohol. By repeating this process two or three times a satisfactory product was obtained. The following is the analysis of three successive fractions.

1. 6.138 mg. substance: 34.673 mg. ammonium phosphomolybdate (Pregl)

2. 4.201 " " : 22.845 mg. ammonium phosphomolybdate

3. 3.782 " " : 20.967 " " "

1. 0.1000 gm. substance: 0.0644 gm. BaSO<sub>4</sub>

2. 0.1000 " " : 0.0656 " "

3. 0.1000 " " : 0.0630 " "

C<sub>5</sub>H<sub>8</sub>O<sub>8</sub>PBa. Calculated. P 8.48, Ba 37.59

Found. 1. " 8.20, " 37.89

2. " 7.92, " 38.60

3. " 8.05, " 37.07

*Method II*—The barium was quantitatively removed with sulfuric acid from barium ribosephosphate which was then converted into the dibrucine salt. The solution of brucine salt was filtered and evaporated to a small volume under reduced pressure. The brucine was removed from the precipitated salt by washing with chloroform. The residue was then recrystallized three or four times from 75 per cent acetone-water solution. The analysis of



the main fraction corresponded with that required for dibrucine ribosephosphate. It had the following composition.

4.635 mg. substance: 9.245 mg. ammonium phosphomolybdate  
 6.1300 " " : 0.301 cc. N (759 mm. and 28°)  
 $C_{11}H_{13}O_{10}N_4P$ . Calculated. P 3.04, N 5.50  
 Found. " 2.89, " 5.55

Its rotation was

$$[\alpha]_D^{25} = \frac{-0.68^\circ \times 100}{1 \times 2.04} = -33.3^\circ \text{ (in pyridine-water, 1:1 by volume)}$$

The dibrucine ribosephosphate in methyl alcohol was decomposed with barium hydroxide. After removal of methyl alcohol the brucine was removed by filtration and the barium salt precipitated with absolute alcohol. This salt was redissolved in water, made just alkaline with barium hydroxide, centrifuged to remove a precipitate, and reprecipitated with methyl alcohol. The analysis of the dried salt corresponded with that required for barium ribosephosphate.

4.390 mg. substance: 26.460 mg. ammonium phosphomolybdate  
 0.1000 gm. " : 0.0644 gm.  $BaSO_4$   
 $C_8H_7O_5PBa$ . Calculated. P 8.48, Ba 37.59  
 Found. " 8.73, " 37.89

*Preparation of Barium Phosphoribonate*—Crude barium ribosephosphate was oxidized with barium hypiodite following the procedure of Levene and Raymond.<sup>11</sup> After oxidation, the solution of phosphoribonic acid, which had been freed of barium and sulfate ions, was rendered neutral with a methyl alcoholic solution of brucine. On standing in the refrigerator, excess brucine crystallized out and was filtered off. On evaporation under reduced pressure, more brucine separated. Finally, the brucine salt separated and was recrystallized first from methyl alcohol and then from 90 per cent methyl alcohol. Dibrucine phosphate is very soluble in methyl alcohol and would remain in the mother liquors. The analysis of the main fraction corresponded with that required for the tribrucine salt of phosphoribonic acid with no water of crystallization.

<sup>11</sup> Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **91**, 751 (1931).

4.385 mg. substance: 7.420 mg. ammonium phosphomolybdate  
 0.8090 " " : 0.430 cc. N (769 mm. and 23.5°)  
 $C_7H_{10}O_{21}N_8P$ . Calculated. N 5.89, P 2.17  
 Found. " 6.17, " 2.45

This brucine salt was converted to the trisodium salt of phosphoribonic acid in the following way. A solution containing 4.56 gm. (0.0032 mol) of the tribrucine salt in methyl alcohol was treated with exactly 0.0096 equivalent of 0.1 N sodium hydroxide. On standing, brucine crystallized out and was filtered off. On evaporation under reduced pressure, further crops of brucine separated and were removed by filtration. The solution was evaporated to a thick syrup, after which it was triturated with methyl alcohol. It became perfectly granular on grinding with fresh portions of methyl alcohol. The salt was dried and analyzed for phosphorus.

4.765 mg. substance: 31.175 mg. ammonium phosphomolybdate  
 4.516 " " : 31.040 " " "  
 $C_5H_8O_9PNa_3$ . Calculated. P 9.93  
 Found. " 9.50  
 " 9.98

Calcium phosphoribonate (prepared from inosinic acid by Levene and Jacobs<sup>3</sup>) was converted to the sodium salt by shaking with a solution of sodium oxalate. In order to preclude the possibility of the presence of sodium oxalate the calcium salt was added in very small quantities until the solution showed a very slight excess of calcium. The solution of sodium salt was then evaporated and granulated with methyl alcohol as described before. It was analyzed for phosphorus.

4.564 mg. substance: 30.045 mg. ammonium phosphomolybdate  
 $C_5H_8O_9PNa_3$ . Calculated. P 9.93  
 Found. " 9.56

*Rotations and Rates of Hydrolysis of Isomeric Phosphoribonic Acids*—The isomeric phosphoribonic acids obtained from the two salts described above were compared as regards their lactonization and the rates of their hydrolysis. The results are given in Tables I and II. In the first and second samples, 0.2574 gm. of dry salt was dissolved in 5 cc. of solution which contained 2.5 cc. (3 equivalents) of 1 N hydrochloric acid. This quantity of salt was

taken because it was equivalent to 0.25 gm. of the calcium salt, the rotation of which had been previously reported by Levene and Jacobs<sup>3</sup> and Levene and Mori.<sup>4</sup> In the third sample, 0.2444 gm. of sodium salt was dissolved in 5 cc. of solution containing 1.57 cc. (2 equivalents) of 1 N hydrochloric acid. The readings were taken immediately in a jacketed 1 dm. tube at 7° by Dr. Alexandre

TABLE I  
*Rotations of Phosphoribonic Acids*

Sample 1 From inosinic acid			Sample 2 From xanthylic acid			Sample 3 From xanthylic acid. Sodium salt + 2 equivalents of acid		
Time	$\alpha^{25}$	$[\alpha]_{5.461}^{25}$	Time	$\alpha^{25}$	$[\alpha]_{5.461}^{25}$	Time	$\alpha^7$	$[\alpha]_{5.461}^7$
<i>hrs.</i>			<i>hrs.</i>			<i>min.</i>		
0	-0.535	-10.42	0	-0.311	-6.05	0	-0.353	-7.22
1	-0.480	-9.34	0.7	-0.319	-6.20	5	-0.350	-7.15
2.1	-0.436	-8.47	2.1	-0.320	-6.21	15	-0.353	-7.22
4.6	-0.318	-6.18	4.4	-0.325	-6.31	45	-0.353	-7.22
22.4	-0.080	-1.55	22.3	-0.352	-6.85	1097	-0.357	-7.29
			28.5	-0.348	-6.76			

TABLE II  
*Hydrolysis of Phosphoribonic Acids*

From inosinic acid		From xanthylic acid	
Time	Hydrolysis	Time	Hydrolysis
<i>hrs.</i>	<i>per cent</i>	<i>hrs.</i>	<i>per cent</i>
0	6.35	0	6.85
1	12.6	1	22.5
2	17.0	2	33.0
4	24.8	4	53.1
6	34.1	6	76.1
11.5	46.6	11.5	79.7

Rothen. The light source was the green line, 5.461 Å., of the mercury vapor lamp.

0.5 cc. portions of Samples 1 and 2 in Table I were diluted to 50 cc. in volumetric flasks and the phosphorus determined colorimetrically.<sup>9</sup> These solutions then contained a total of 0.051 mg. of phosphorus per cc. The acid concentration was 0.01 N, which

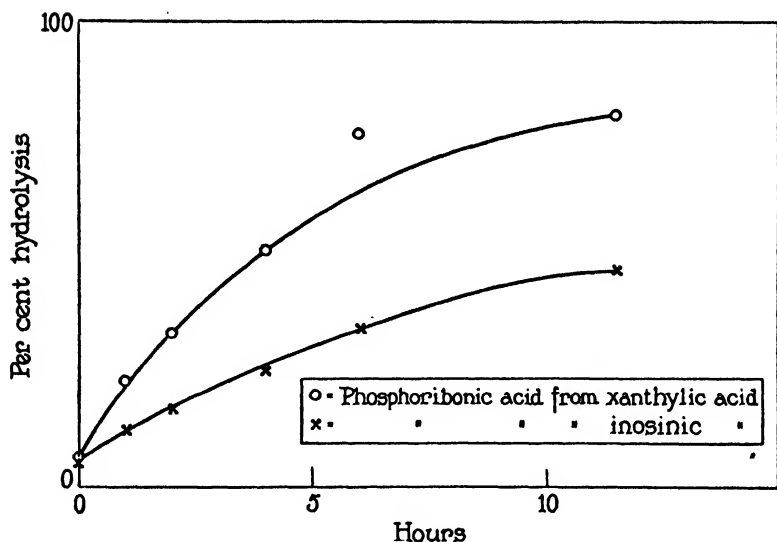


FIG. 1. Rates of phosphate hydrolysis of the phosphoribonic acids

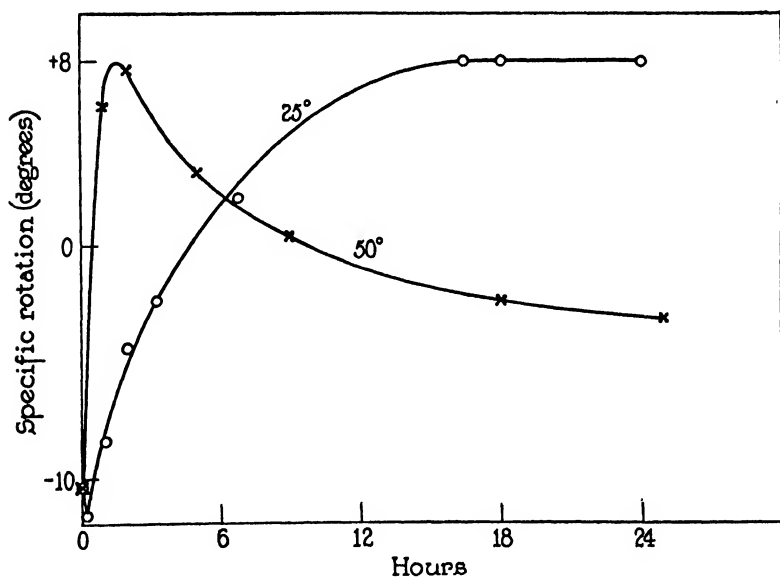


FIG. 2. Glycoside formation of ribosephosphoric acid from xanthylic acid.

was about equal to that of the free phosphoribonic acid. 5 cc. portions were sealed in test-tubes and hydrolyzed in a boiling water bath for different periods of time. The results<sup>9, 10</sup> are recorded in Table II and Fig. 1. It is concluded from the results of lactone formation and rates of hydrolysis that the phosphoribonic acids, obtained from xanthylic and inosinic acids respectively, are different.

*Glycosides of Ribosephosphoric Acid*—The glycosides were prepared according to the directions of Levene and Raymond.<sup>12</sup> A solution of ribosephosphoric acid was prepared by dissolving 2.5 gm. of barium ribosephosphate in 100 cc. of methyl alcohol containing 1 gm. of dry hydrogen chloride. The changes in specific rotation of the solutions were observed at 25° and 50°. Fig. 2 shows curves which are typical of the formation of both a <1, 4> and a <1, 5> glycoside. This glycoside formation showed that in the ribosephosphoric acid from xanthylic acid both the (4) and (5) positions are free.

<sup>12</sup> Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **89**, 479 (1930).

## THE REACTION RATE OF POTASSIUM IODIDE WITH DIBROMIDES OF THE ETHYLENE BROMIDE TYPE

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### INTRODUCTION

In a method for analyzing mixtures of the three normal butenes it was necessary<sup>1</sup> to utilize the specific reaction rate constants of the corresponding dibromobutanes with potassium iodide in methanol at 75°. The stoichiometric equation involved was expressed by equation (1)



Kinetically the reaction was readily followed by the titration of the iodine. It was pointed out in this article that the equation could equally well, and more probably should, have been expressed as follows



which differs from the first form only by equation (3)



In an aqueous solution this reaction of iodine with potassium iodide takes place completely; but in an alcoholic solution this is perhaps questionable and for this reason the first form of the dibromide reaction was used in the previous paper. In this article, however, are given calculations made from the experimental data of van Duin<sup>2</sup> which show that equation (3) is evidently complete even in alcoholic solutions and, therefore, that one mole of dibromide<sup>3</sup> reacts with three

<sup>1</sup> Dillon, Young and Lucas, THIS JOURNAL, 52, 1953 (1930).

<sup>2</sup> Van Duin, *Rec. trav. chim.* 43, 341 (1924).

<sup>3</sup> The term "dibromide" as used herein is understood to mean only ethylene bromide and its homologs, or their substituted derivatives.

moles of potassium iodide according to equation (2). Hence this stoichiometric equation should be the basis for the differential equations expressing the velocity of the reaction.

However, the use of either equation would not alter the result previously found, namely, that kinetically the reaction is one of the second order, the rate being proportional to the first power of the concentrations of both the dibromobutane and the potassium iodide.<sup>4</sup> This is in accord with the results of Biilmann,<sup>5</sup> who found that the reaction of 2,3-dibromopropanoic acid with potassium iodide in dilute sulfuric acid at 25° was also of the second order. In addition it may be mentioned that Slator<sup>6</sup> has found that the reactions of ethylene bromoiodide and ethylene iodide with potassium iodide are very rapid. From this it follows that the comparatively slow reaction of ethylene bromide, presumably involving either one or both of the preceding compounds as an intermediate product, would not be of the third order, but of the first or second. On the other hand, van Duin<sup>2,7</sup> discusses the work of Biilmann and Slator, rejects their conclusions and reports that the reaction of dibromides<sup>8</sup> with potassium or sodium iodide is of the third order.

For this reason, it was deemed desirable to extend somewhat the investigations previously described. The work was aided financially by a grant made to Professor A. A. Noyes by the Carnegie Institution of Washington. The author is indebted to Professor Howard J. Lucas for his valuable criticisms and suggestions.

#### EXPERIMENTAL

The procedure and technique used in the rate measurements are as described in the previous work.<sup>1</sup> To this description the following further details should be added. The purified ethylene and propylene

<sup>4</sup> Since the potassium iodide concentration is seven to twelve times that of the dibromide, calculation of the second order constant according to either stoichiometric equation leads to substantially the same numerical value of the constant. (See Table IV.)

<sup>5</sup> Biilmann, *Rec. trav. chim.*, **36**, 319 (1917).

<sup>6</sup> Slator, *J. Chim. Soc.*, **85**, 1697 (1904).

<sup>7</sup> Van Duin, *Rec. trav. chim.*, **45**, 345 (1926).

<sup>8</sup> Van Duin tested the order only with ethylene bromide but his conclusions were generalized for all dibromides.

bromide boiled, respectively, at 130.3–130.5° and 140.0° at 743–744 mm. The small corrections for the disappearance of iodine due to its reaction with impurities remaining in the purified alcohol solvents were made with greater accuracy for the experiments involving short intervals of time. For the preparation of the reaction mixtures the dried potassium iodide was weighed into the volumetric flask and the salt completely dissolved by shaking with almost the required volume of solvent. The solution was brought to a temperature of 20° in a thermostat, the required amount of dibromide was added from a weighing pipet and the solution quickly made up to the exact volume. The solution was next pipetted into the reaction tubes. They were then sealed, placed in a light-tight thermostat which was regulated to within  $\pm 0.03^\circ$  of the desired temperature, removed at appropriate intervals and analyzed by titration with 0.02 *N* thiosulfate.<sup>9</sup> The second and third order specific reaction rate constants were calculated from the analytical data by means of the usual integrated expressions for  $k_2$  and  $k_3$ .<sup>9</sup> They are, on the basis of equation (2)

$$k_2 = \frac{2.303}{t(a - 3b)} \log_{10} \frac{b(a/b - 3\varphi)}{a(1 - \varphi)} \quad (4)$$

$$k_3 = \frac{1}{t(a - 3b)^2} \left[ -\frac{3\varphi(a - 3b)}{a(a/b - 3\varphi)} + 2.303 \log_{10} \frac{b(a/b - 3\varphi)}{a(1 - \varphi)} \right] \quad (5)$$

where  $t$  is the time in hours,  $a$  and  $b$  are the initial concentrations of potassium iodide and dibromide, respectively, in moles per liter at 20° and  $\varphi$  is the fraction of the dibromide which had reacted at the time  $t$ . The values of  $k_2$  and  $k_3$  are thus expressed in (hours)<sup>-1</sup> (moles/liter)<sup>-1</sup> and (hours)<sup>-1</sup> (moles/liter)<sup>-2</sup>, respectively.

The times involved in the rate measurements were always taken from the time of immersion of the tubes in the thermostat. The reactions were in all cases so slow at 20° that the interval between the addition of the dibromide and the insertion of the tubes into the thermostat, namely, twenty to thirty minutes, was entirely negligible. The concentration of the dibromides was kept essentially constant throughout the work since the rate is undoubtedly proportional to the first

<sup>9</sup> The expression for  $k_3$  in the preceding article<sup>1</sup> (equation 17) is incorrectly printed, the logarithm applying only to the first term within the brackets.



power of this value.<sup>1, 2, 5</sup> Measurements were made with at least two different initial concentrations of the potassium iodide in order to determine the dependence of the reaction rate upon the concentration of this reactant.

TABLE I

*Influence of Triiodide Formation on the Reaction Rate of  $p\text{-NaSO}_3\text{C}_6\text{H}_4\text{CHBr-CHBrCOONa}$  with Potassium Iodide<sup>a</sup>*

KI, 0.25 <i>M</i> Dibromide, 0.025 <i>M</i>	Time, min.	Constants $\times 10^3$		
		$k_1^b$	$k_2^c$	$k_3^d$
A. Without initial iodine	931	3.03	0.742	0.749
	1721	3.06	.741	.752
	2586	3.08	.718	.739
	3856	2.99	.705	.727
	Average	3.04	0.727	0.742
B. With initial iodine ( $\cong 0.1$ initial KI)	932	2.75	0.675	0.757
	1724	2.71	.659	.745
	2587	2.68	.644	.735
	3858	2.58	.613	.705
	Average	2.68	0.648	0.734
Difference between A and B, %.....		12.6	11.5	1.1

<sup>a</sup> These experimental data were taken from the work of van Duin (Ref. 2).

<sup>b</sup> On the basis of equation (1):

$$k_3 = \frac{1}{t(a - 2b)^2} \left[ -\frac{2\varphi(a - 2b)}{a(a/b - 2\varphi)} + 2.303 \log_{10} \frac{b(a/b - 2\varphi)}{a(1 - \varphi)} \right]$$

The values were calculated and reported by van Duin.

<sup>c</sup> On the basis of equation (1)

$$k_2 = \frac{1}{t(a - 2b)} \log_{10} \frac{b(a/b - 2\varphi)}{a(1 - \varphi)}$$

<sup>d</sup> On the basis of equation (2):  $k_2$  from equation (4).

## RESULTS AND DISCUSSION

*Influence of Iodine upon the Rate of Reaction.*—This influence is shown in Table I. Thus, when either second or third order constants are calculated on the basis of the stoichiometric equation (1) the pres-

ence of some initial iodine in the reaction mixture leads to lower values of the constants than in the absence of this initial iodine (*e.g.*, 2.68 and 0.648 as against 3.04 and 0.727 without the iodine). As may be seen, these values differ by approximately 10% which corresponds with the decrease in iodide ion concentration brought about by the introduction of iodine into the reaction mixture in experiment B. However, when second order constants are calculated on the basis of equation (2) in which the formation of triiodide ion is taken into account, the values are practically identical (*e.g.*, 0.742 and 0.734). It is perhaps needless to say that in these last calculations a correction is necessary for the effect of this initial iodine upon the concentration of the iodide ion. The results indicate that equation (2) is the correct stoichiometric form to use in the calculation of the specific rate constants for this reaction.

*Order and Temperature Coefficients of the Reaction Rates.*—The detailed data relating to the reaction rate measurements of ethylene bromide with potassium iodide in methanol at 30.00, 59.72 and 74.93° are given in Table II. Table III gives the data for propylene bromide at 59.72 and 74.93°. The values of  $k_2$  and  $k_3$  were calculated from equations (4) and (5), respectively. The same terms and units are used in these tables as indicated previously for these two equations except that the concentrations of potassium iodide and dibromide are indicated by KI and  $C_2H_4Br_2$  (or  $C_3H_6Br_2$ ) instead of by  $a$  and  $b$ . As is evident, there is good agreement among the second order values both in individual experiments and in ones with different initial iodide concentrations, whereas the third order values show wide deviations.

Since the preceding section indicates that equation (2) is the correct one for these reactions in alcoholic solutions, this same method of calculation has been applied to the data previously published on the dibromobutanes.<sup>1</sup> The values of the reaction constants for all of the compounds investigated are summarized in Table IV. The table includes rate measurements of the reaction carried out in aqueous methanol and ethanol solutions in order to determine possible effects arising from the addition of water to the methanol solvent and from the substitution of ethanol for methanol. It is evident that at least as far as non-ionic dibromides are concerned there is no doubt that the reaction is of the second order.

Two interesting comparisons can be drawn from the values of  $k_2$

collected in Table IV. They are, first, at a given temperature the rates of reaction for all of the dibromides investigated are of the same

TABLE II  
*Reaction Rate Constants of Ethylene Bromide with Potassium Iodide in 99% Methanol*

Temp. 30.00°							
KI, C <sub>2</sub> H <sub>4</sub> Br <sub>2</sub> , Time	0.2152 M 0.02450 M φ	k <sub>2</sub>	k <sub>3</sub>	KI, C <sub>2</sub> H <sub>4</sub> Br <sub>2</sub> , Time	0.1469 M 0.02529 M φ	k <sub>2</sub>	k <sub>3</sub>
91.4	0.1757	0.0101	0.0484	90.7	0.1191	0.0099	0.0702
100.8	.1925	.0102	.0490	100.0	.1318	.0103	.0711
115.3	.2154	.0101	.0490	114.5	.1485	.0100	.0712
126.2	.2340	.0102	.0496	125.4	.1610	.0100	.0715
136.9	.2498	.0102	.0497	136.2	.1750	.0101	.0728
151.5	.2719	.0102	.0500	150.8	.1911	.0101	.0731
174.4	.3061	.0103	.0508	173.7	.2163	.0102	.0740
Average		0.0102	0.0495	Average		0.0101	0.0720

Temp. 59.72°							
KI, C <sub>2</sub> H <sub>4</sub> Br <sub>2</sub> , Time	0.2237 M 0.02655 M φ	k <sub>2</sub>	k <sub>3</sub>	KI, C <sub>2</sub> H <sub>4</sub> Br <sub>2</sub> , Time	0.1531 M 0.02864 M φ	k <sub>2</sub>	k <sub>3</sub>
8.25	0.3985	0.299	1.46	8.25	0.2863	0.292	2.10
11.25	.4914	.299	1.49	11.25	.3630	.296	2.19
13.25	.5420	.297	1.50	13.25	.4099	.299	2.26
15.25	.5909	.299	1.54	15.50	.4572	.302	2.33
17.25	.6332	.301	1.57	17.25	.4890	.303	2.37
20.25	.6823	.298	1.57	20.25	.5396	.305	2.46
23.25	.7268	.298	1.61	23.25	.5795	.302	2.49
Average		0.299	1.53	Average		0.300	2.32

Temp. 74.93°			
KI, C <sub>2</sub> H <sub>4</sub> Br <sub>2</sub> , Time	0.2157 M 0.02200 M φ	k <sub>2</sub>	k <sub>3</sub>
1.00	0.2347	1.29	...
1.25	.2884	1.32	...
1.67	.3661	1.35	...
3.00	.5564	1.39	...
		1.34	

order of magnitude, with the exception of ethylene bromide, which reacts much faster than the others, and, second, for a given compound the reaction rate is greater in the mixed solvent which has the higher molal fraction of water.

TABLE III  
*Reaction Rate Constants of Propylene Bromide with Potassium Iodide in 99% Methanol*

Temp. 59.72°							
KI, C <sub>3</sub> H <sub>5</sub> Br <sub>2</sub> , Time	0.2208 M 0.02216 M φ	k <sub>1</sub>	k <sub>2</sub>	KI, C <sub>3</sub> H <sub>5</sub> Br <sub>2</sub> , Time	0.1827 M 0.02315 M φ	k <sub>1</sub>	k <sub>2</sub>
17.50	0.04365	0.0118	0.0540	21.00	0.04331	0.0116	0.0641
22.08	.05381	.0116	.0531	25.00	.05110	.0115	.0640
25.50	.06175	.0116	.0530	30.83	.06150	.0114	.0630
31.00	.07306	.0113	.0520	35.83	.06648	.0106	.0590
41.75	.09432	.0110	.0505	45.25	.08337	.0107	.0595
46.32	.1031	.0109	.0502	51.00	.09117	.0105	.0582
51.58	.1133	.0108	.0499	57.00	.1018	.0104	.0587
56.58	.1228	.0107	.0497	66.00	.1286	.0117	.0658
66.00	.1441	.0110	.0509				
Average 0.0113 0.0515				Average 0.0111 0.0615			
Temp. 59.72°				Temp. 74.93°			
KI, C <sub>3</sub> H <sub>5</sub> Br <sub>2</sub> , Time	0.1491 M 0.02444 M φ	k <sub>1</sub>	k <sub>2</sub>	KI, C <sub>3</sub> H <sub>5</sub> Br <sub>2</sub> , Time	0.2170 M 0.02404 M φ	k <sub>1</sub>	k <sub>2</sub>
21.00	0.03651	0.0118	0.0788	17.87	0.1952	0.0580	..
25.00	.04143	.0114	.0773	20.50	.2179	.0575	..
30.83	.04861	.0109	.0742	26.08	.2688	.0581	..
35.83	.05537	.0108	.0736	31.00	.3105	.0586	..
45.25	.06911	.0108	.0736	42.05	.3907	.0585	..
51.00	.07670	.0107	.0731	56.75	4.800	.0584	..
57.00	.08511	.0107	.0734				
66.00	.1017	.0111	.0769				
Average 0.0110 0.0751				Average 0.0582			

In Table V are given the values of the heats of activation  $Q$ , and the temperature coefficients for 10°, calculated according to the Arrhenius equation from the rate constants at 59.72 and 74.93°. It is interesting that the temperature coefficients of 1,2-dibromopropane and

1,2-dibromobutane are practically identical, a result not unexpected in view of their similarity in structure.

TABLE IV  
Summary of Reaction Rate Constants

Temp., °C.	Dibromide ( $\approx 0.022 M$ )	KI concn. ( $M$ )	Solvent wt. per cent. of methanol	Specific reaction rate constants		
				$k_1$	$k_2$	
30.00	$\text{CH}_2\text{BrCH}_2\text{Br}$	0.2152	99.2	0.0102	0.0495	
		.1469	99.2	.0101	.0720	
		.2207	63.0	.0154		
		.1496	63.0	.0157		
59.72	$\text{CH}_2\text{BrCH}_2\text{Br}$	.2237	99.0	.299	1.53	
		.1531	99.0	.300	2.32	
		.2240	72.0	.402		
		.1516	72.0	.411		
	$\text{CH}_3\text{CHBrCH}_2\text{Br}$	.2208	99.0	.0113	0.0515	
		.1827	99.0	.0111	.0615	
		.1491	99.0	.0110	.0751	
		.2194	72.0	.0166		
		.1524	72.0	.0169		
		.2225	68.3 <sup>a</sup>	.0261		
		.1519	68.3 <sup>a</sup>	.0260		
		.2281	99.0	.0148 (0.0147) <sup>b</sup>		
	$\text{CH}_3\text{CHBrCHBrCH}_3$ (meso)	.2287	99.0	.00902 (.00896) <sup>b</sup>		
	(racemic)	.2281	99.0	.00465 (.00462) <sup>b</sup>		
74.93	$\text{CH}_2\text{BrCH}_2\text{Br}$	.2157	99.0	1.34		
	$\text{CH}_3\text{CHBrCH}_2\text{Br}$	.2170	99.0	.0582		
	$\text{CH}_3\text{CH}_2\text{CHBrCH}_2\text{Br}$	.2298	99.0	.0800 (.0773) <sup>b</sup>	0.357 <sup>c</sup>	
		.2296	99.0	.0787		
	$\text{CH}_3\text{CHBrCHBrCH}_3$ (meso)	.1470	99.0	.0811 (.0784) <sup>b</sup>	.567 <sup>c</sup>	
		.2217	99.0	.0562 (.0544) <sup>b</sup>	.256 <sup>c</sup>	
		.1461	99.0	.0564 (.0545) <sup>b</sup>	.386 <sup>c</sup>	
		(racemic)	.2327	99.0	.0301 (.0294) <sup>b</sup>	.130 <sup>c</sup>
		(racemic)	.2273	99.0	.0301	
		(racemic)	.1498	99.0	.0310 (.0306) <sup>b</sup>	.209 <sup>c</sup>

<sup>a</sup> Wt. per cent. ethanol. <sup>b</sup> Previously reported value (Ref. 1); see footnote c, Table I. <sup>c</sup> Previously reported value (Ref. 1); see footnote b, Table I.

*Recalculation of van Duin's Results.*—The third order specific rate constants reported by van Duin<sup>2,7</sup> are not the same for different initial

concentrations of potassium iodide. It may be readily seen from the results that second order constants would be in much better agreement. It suffices to say that the values which have been recalculated, while they do not agree as well as those given in Table IV, obviously indicate that van Duin was in error when he assumed that the rates were of the third order.

*Influence of Ionic Strength upon the Reaction Rates.*—The Brönsted theory<sup>10</sup> of the velocity of ionic reactions is well known and has been applied extensively. The effect of the ionic strength upon the classical second order specific rate constant is indicated by the relation  $k_2 = k_0 10^{z_a z_b \sqrt{\mu}}$  which holds strictly only in dilute solution. How-

TABLE V

*Heats of Activation and Temperature Coefficients for Reactions of Dibromides with Potassium Iodide in 99% Methanol*

Dibromide	$k_2$		Heat of activation, cal.	Temperature coefficient for 10°	
	59.72°	74.93°		$\frac{69.72^\circ}{59.72^\circ}$	$\frac{74.93^\circ}{64.93^\circ}$
CH <sub>2</sub> BrCH <sub>2</sub> Br.....	0.300	1.34	22700	2.73	2.64
CH <sub>3</sub> CHBrCH <sub>2</sub> Br.....	.0111	0.0582	25100	3.02	2.92
CH <sub>3</sub> CH <sub>2</sub> CHBrCH <sub>2</sub> Br.....	.0148	.0800	25500	3.09	2.98
CH <sub>3</sub> CHBrCHBrCH <sub>3</sub> (meso).....	.00902	.0563	27700	3.39	3.27
CH <sub>3</sub> CHBrCHBrCH <sub>3</sub> (racemic).....	.00465	.0304	28400	3.50	3.37

ever, even in the relatively concentrated solutions in which the rate measurements of the reaction between dibromides and iodide ion have all been made, the effects predicted by the Brönsted expression may be qualitatively demonstrated. Van Duin measured the rates of reaction of potassium and sodium iodide with dibromides capable of undergoing ionization and found an increase in the specific reaction rate with an increase in concentration of iodide ion or on the addition of various salts to the reaction mixture. Incidentally, the result was not ascribed to the Brönsted effect. The reaction of potassium and so-

<sup>10</sup> Brönsted, *Z. physik. Chem.*, **102**, 169 (1922); "Theory of the Velocity of Ionic Reactions" in the Columbia University Lectures, "Contemporary Developments in Chemistry," Columbia University Press, March, 1927; see also, Brönsted and Livingston, *THIS JOURNAL*, **49**, 435 (1927).

dium iodide with a non-ionizable dibromide, namely, ethylene bromide, has been carried out in this work and in this case the rate was found to decrease only slightly on the addition of salts. Thus, in the case where both reactants are ionic and are of the same sign, the reaction rate increases with ionic strength, but where one of the reactants is electrically neutral, the ionic strength is without effect on the rates. The results are summarized in Table VI.

TABLE VI

*Influence of Ionic Strength on the Reaction Rates of Dibromides with Potassium Iodide*

	Molal concn.		Added substance	$\mu$	$k_2 \times 10^3$
	KI	Added substance			
A. Ionized dibromide, <sup>a</sup> $p$ -NaSO <sub>3</sub> C <sub>6</sub> H <sub>4</sub> -CHBrCHBrCOONa, 0.025 <i>M</i>	1.0	....	....	1.07	1.10
	2.0	....	....	2.07	1.49
	1.0	1.0	KBr	2.07	1.54
	1.0	1.0	KCl	2.07	1.57
	1.0	0.5	CaCl <sub>2</sub>	2.57	1.73
B. Un-ionized dibromide, <sup>b</sup> CH <sub>2</sub> Br-CH <sub>2</sub> Br, 0.022 <i>M</i>	0.22	....	....	0.22	80.0
	.22	0.11	KCl	.33	77.9
	.22	.22	KBr	.44	72.5
C. Undissociated dibromo acid, <sup>c</sup> CH <sub>2</sub> -BrCHBrCOOH, 0.025 <i>M</i>	.5	.125	HCl	.63	0.343
	1.0	.125	HCl	1.13	.317
	2.0	.250	HCl	2.25	.364
	2.0	.500	HCl	2.50	.354

<sup>a</sup> These results were calculated from the data of van Duin (Ref. 2). Similar results may be calculated from his data for CH<sub>2</sub>BrCHBrCOONa. <sup>b</sup> These results were obtained in this work: temp. 40°; solvent, 60% ethanol. <sup>c</sup> Also, recalculated from data of van Duin (Ref. 2). Other acids gave the same results.

In addition it should be pointed out that the rates of reaction of dibromo acids with potassium iodide as reported in Table VI are not affected by altering the ionic strength of the solutions by changing the concentration of either the potassium iodide or the added hydrochloric acid. This is logical since the amount of hydrochloric acid present in these reactions should be sufficient to depress completely the dissociation of these weak organic acids so that in their reactions with iodide ion they conform to the case where one of the reactants is elec-

trically neutral. It is not surprising, however, that the specific rate constant of 2,3-dibromopropanoic acid under the various conditions tabulated (Table VI) differs from the rate<sup>11</sup> when there is no free hydrochloric acid present. In this last instance partial dissociation of the organic acid undoubtedly occurs.

#### SUMMARY

The reaction rates of potassium iodide with ethylene bromide and propylene bromide in methanol have been measured at 30, 60 and 75° and found to be of the second order. From the influence of iodine upon the rate it has been shown that in alcoholic solutions one mole of dibromide reacts with three moles of potassium iodide.

Previously reported second order constants for the reaction of the butylene bromides have been recalculated on this basis. From these rate constants, the heats of activation and temperature coefficients for ethylene bromide, propylene bromide and the three normal butylene bromides have been calculated.

Recalculation of van Duin's results shows that the reactions of probably all dibromides with potassium iodide are second order and not third order as he claimed.

An increase in the ionic strength of the reaction medium by the addition of neutral salts slightly decreases the reaction rate of the non-ionic dibromides with iodide ion. On the other hand, under these conditions the reaction rate is appreciably increased in the case of ionized dibromides as the Brönsted theory demands.

Ref. 2, p. 351:  $k_1 = 0.123 \times 10^{-3}$ ,  $k_2 = 0.246 \times 10^{-3}$ .





## A METHOD FOR WASHING CORPUSCLES IN SUSPENSION

By C. A. LINDBERGH

*(From the Laboratories of The Rockefeller Institute for Medical Research)*

The original fluid may be replaced and corpuscles washed while still in suspension during centrifugation. This is accomplished by placing the suspension in a conical chamber (A). During centrifugation, the replacement fluid is introduced at the outer and narrow end of the chamber while the replaced fluid is forced out at the inner and wider end. The chamber is so designed that, when properly operated, the rate of flow at the narrow end is too rapid to permit the settling and packing of the corpuscles, while at the wide end it is too slow to carry them out with the replaced fluid. The corpuscles, therefore, remain suspended in the middle of the chamber.

### *Technique*

The conical chambers (A) are filled and inserted in the centrifuge head. These are so designed that all connections are made automatically as they are placed in their guides. An injector tube (G) from an inverted flask (I) containing the replacement solution is clamped in position in the center of the intake chamber (E). The position of the mouth of this tube regulates the height of fluid in the intake chamber.<sup>1</sup> A clamp on the rubber connection (H) to the flask (I) prevents the fluid from flowing out. The centrifuge is started and run at full operating speed long enough to permit the corpuscles to settle away from the outlet of the chamber (A). The clamp on the flask connection

<sup>1</sup> When only one conical chamber is used or when accurate distribution is not essential, the jets (F) may be replaced by full sized glass tubes and the rates of flow of replacement fluid regulated by a jet or valve before it is introduced into the intake chamber. In this case it is convenient to keep the mouth of the injector tube close to the top of the intake chamber. When one of the conical chambers is not in use the jet is replaced with a closed tube and the chamber filled with fluid of proper density to maintain balance.



is then removed and the replacement fluid permitted to flow into the intake chamber. The rate of flow to each conical chamber is regulated by the size of the jets (F) leading to them. The overflow is carried to a collector pan below the centrifuge head. When the desired washing or dilution has been obtained, the flow of replacement fluid is cut off and the centrifuge stopped by applying the brake. The conical chambers are removed from their guides and the suspension of corpuscles may be poured out through the vent tube (C). The corpuscles come in contact with glass only. All vessels and tubes which come in contact with the replacement fluid are glass and their connections rubber.

The rate of replacement and washing depends upon the suspension to be washed and the centrifugal force used. In a first test, a 90 cc solution containing corpuscles in suspension and .02 per cent. phenol red was centrifuged at approximately 650 times gravity at the maximum cross section of the conical chamber. After one minute, the replacement fluid was permitted to flow through the chamber at the rate of 60 cc per minute. At the end of fifteen minutes, the dilution of the indicator showed that only a fraction of 1 per cent. of the original fluid still remained.



## THE RACES THAT CONSTITUTE THE GROUP OF COMMON FIBROBLASTS

### I. THE EFFECT OF BLOOD PLASMA

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PLATES 26 to 28

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Since fibroblasts from the heart of the embryo chick were first isolated in pure cultures, it has been known that they multiply in embryonic tissue juice and in fluids containing the larger protein split-products.<sup>1</sup> Adult blood plasma, although the optimal medium for the cultivation of blood and tissue macrophages, was found to be insufficient as a source of nutriment for the heart cells.<sup>2</sup> Later work<sup>3</sup> showed, however, that fibroblasts derived from another part of the organism behaved differently. Thus, morphologically similar cells, isolated from the perichondrium of cartilage, multiplied at a slow but uniform rate when treated with blood plasma and could be kept in good condition over very long periods of time. This led to the discovery<sup>4</sup> that various races of fibroblasts could be isolated simultaneously from the same organism, and that each race, according to its origin, constituted a specific cell type characterized solely by its nutritional properties. Those cell types which exhibited the highest residual growth energy, as determined by their capacity to proliferate in a medium free from nitrogenous food substances, were able not only to subsist on very minute quantities of embryonic tissue juice present in the medium, but also to live on plasma alone. The present communication devotes itself to a consideration of the nutritional properties of fibroblasts with special reference to the effect of blood plasma on their multiplication and behavior *in vitro*.

<sup>1</sup> Carrel, A., *J. Exp. Med.*, 1912, 15, 516; 1913, 17, 14. Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, 44, 503.

<sup>2</sup> Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, 34, 317; 1923, 37, 653, 759.

<sup>3</sup> Fischer, A., and Parker, R. C., *Arch. exp. Zellforsch.*, 1929, 8, 325.

<sup>4</sup> Parker, R. C., *Arch. exp. Zellforsch.*, 1929, 8, 340.

### *Materials and Procedures*

The material consisted of various pure strains of fibroblasts which were isolated simultaneously from embryo chicks ranging from 13 to 15 days of age. These strains were derived from heart muscle, skeletal muscle, the perichondrium of cartilage, and the periosteum of bone, respectively. Until used for the experiments, which were made in flasks,<sup>5</sup> the strains were carried either by the hanging drop method, or in flasks, on such combinations of plasma and embryonic tissue juice as were favorable for the maximum proliferation of the various cell types. According to the usual procedure, the original explants were very early eliminated and discarded by repeated selection of only the marginal area of outgrowth at the time of transfer, thus insuring against a multiplicity of cell types being carried in the individual strains. Each series of experiments was made from strains of the same age.

The cultures comprising the individual experiments were made and treated as follows: The explants were placed in flasks to which had previously been added 0.3 cc. of chick plasma diluted with twice that amount of Tyrode solution. Coagulation of the plasma clot was allowed to take place spontaneously and without the customary addition of embryonic tissue juice. On the following day, the clots were reinforced by the addition of 0.25 cc. of plasma diluted with an equal amount of Tyrode solution. This was allowed to coagulate of its own accord as before. On the next day, and three times a week thereafter, the cultures were washed for 2 hours with Tyrode solution, after which they were treated with a 50 per cent solution of chick plasma diluted with Tyrode solution. Sufficient heparin<sup>6</sup> had previously been added to the solution in order to prevent its coagulation over a period of 2 hours at 37°C. This mixture was finally aspirated and the cultures were returned to the incubator until the next treatment. Whenever it became necessary to subdivide and transfer the cultures, the subcultures were prepared according to the original procedure, and the same manner of treatment was resumed.

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<sup>5</sup> The flasks used were of Pyrex and included two types designed by Carrel. The first, or the D-3 type bearing the oblique neck, has been described many times and is in general use. The second type is more recent (Carrel, A., *Compt. rend. Soc. biol.*, 1930, 105, 826), and has been designated as the microflask. This new flask is 25 or 30 mm. wide and 5 mm. high (inside dimensions). It has a straight neck which is 5 mm. in diameter. At the union of the chamber with the neck, there is a slight depression in the glass which renders possible the maintenance of 1 or 1.5 cc. of medium in the flask without the danger of the fluid running into the neck. The wall is sufficiently thin to permit examination of the tissue cells with a 3 mm. immersion lens. At the termination of an experiment, the tissues may be fixed and stained *in situ*.

<sup>6</sup> In the concentration used, appropriate experiments have shown that the heparin has no appreciable effect upon the rate of growth of fibroblasts.

Growth curves of the cultures were constructed by the planimetric measurement of outline drawings of the surface area which were made from time to time with the projectoscope. Although the limitations of this method have long since been realized, it remains the only practical one available at the present time.

#### EXPERIMENTS AND RESULTS

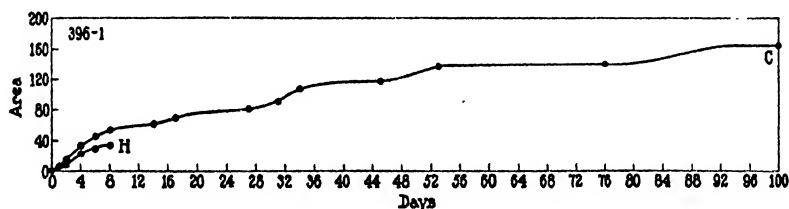
*1. Rate and Mode of Growth of Fibroblasts Grown on a Plasma Medium.*—In view of the marked differences in food requirements already found to exist between the four types of mesenchyme cells employed in the present experiments, one would expect to find differences of the same order in the ability of the various races to multiply on a plasma diet. In general, this is the case. Of the four types, the heart muscle fibroblasts, which invariably have the lowest residual growth energy and the lowest rate of proliferation on a medium containing embryonic tissue juice, multiply least rapidly in the plasma medium. These differences are as great when the different cell types of the same age are cultivated under identical conditions in separate flasks (Fig. 1) as when they are cultivated side by side in the same medium (Text-fig. 1; Figs. 2 and 3). Occasionally, the heart muscle fibroblasts have been found to succumb soon after having been placed on the plasma diet. When, however, the cells survive a certain period of adjustment, they are usually capable of continual proliferation for very long periods of time. Cultures of heart muscle fibroblasts, together with representative cultures from two other cell strains, namely, fibroblasts from skeletal muscle and the perichondrium of cartilage, have been grown on this medium for as long as 236 days, at which time it was necessary to terminate the experiment (Fig. 6).

The change that occurs in the mode of growth of cell colonies transferred from a medium containing embryonic tissue juice to one in which it is absent is not abrupt. Cells are capable of storing food substances during periods of plenty, which reserves they in turn utilize while becoming adjusted to a poorer medium.<sup>7</sup> But, inasmuch as the medium employed in the present experiments is thoroughly washed with Tyrode solution prior to each treatment with fresh plasma, it is not possible that these reserves could suffice for more than a few days.

<sup>7</sup> Carrel, A., *J. Exp. Med.*, 1923, 38, 521.

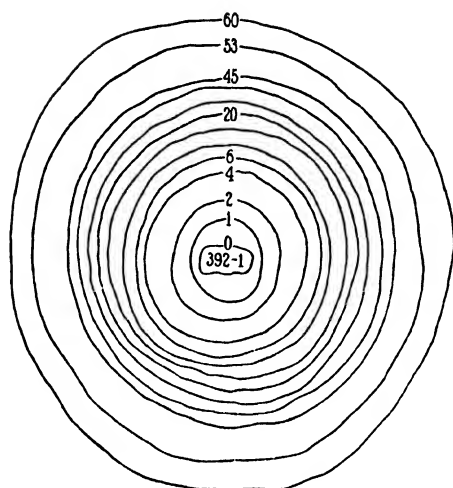


Cultures that are transplanted to the plasma medium acquire, almost immediately, features by which they may readily be distinguished from cultures of the same cell type that have access to embryonic tissue juice. In the plasma medium, cell colonies become very dense (Fig. 4). Even at the periphery, the young cells grow in many layers forming a dense entanglement not unlike fresh tissue removed from the organism. This renders it impossible to state definitely that a culture which has ceased to increase in surface area has also ceased to increase in cell population. In fact, there is abundant evidence that all cultures which cease to grow under these conditions are not necessarily dead. Many of the component cells remain, for very long periods of time, in a quiescent state which is perhaps not unlike that

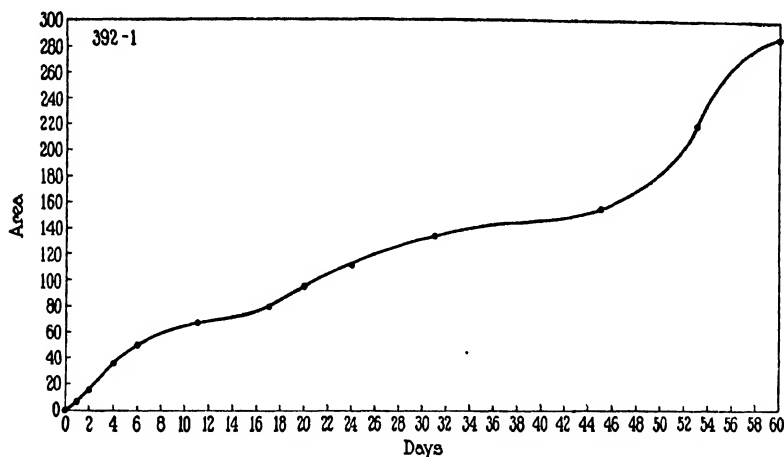


TEXT-FIG. 1. Culture 396-1. Curves showing the rate of growth of cultures of fibroblasts from heart (*H*) and from the perichondrium of cartilage (*C*) cultivated side by side in the same medium for 100 days and subjected to the plasma treatment. The heart fibroblasts ceased to show activity after the 8th day. The fibroblasts from cartilage continued to multiply for 100 days, at which time they were transferred to a fresh medium. (Compare Figs. 2 and 3.)

of the dormant cells in the adult organism. As an example, reference might be made to a culture belonging to a series which, although regularly washed and treated with fresh plasma, was allowed to remain in the original flasks for 100 days (Text-figs. 2 and 3; Fig. 4). During the last 40 days of this period, the culture referred to showed little increase in surface area. Examination of the peripheral areas revealed the presence of many cells in various stages of disintegration but, together with these, large numbers of cells which, although heavily granulated and relatively inactive, were obviously alive. When, however, the culture was subdivided and transferred to a fresh medium, active cell multiplication was resumed. From the very regular distribution of the new cells which grew out from the various transplants



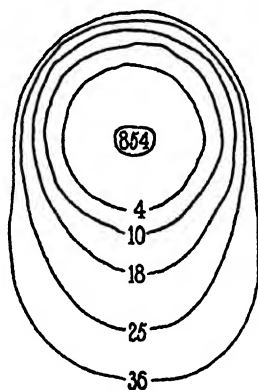
TEXT-FIG. 2. Culture 392-1. Diagrammatic representation of the increase in area of a culture of heart fibroblasts, in the 12th passage, subjected to the plasma treatment. Tracings made from the projected culture at various time intervals from the moment it was placed in the flask until the 60th day. (Compare Fig. 4.)



TEXT-FIG. 3. Culture 392-1. Curve showing the rate of growth of a culture of heart fibroblasts, in the 12th passage, subjected to the plasma treatment. The units of area were ascertained by planimetric measurement of the tracings represented in Text-fig. 2.

(Fig. 5), it may be concluded that living cells had been evenly scattered throughout the whole mass of the original culture. It is of interest to note that this was a culture of heart muscle fibroblasts which had been subdivided and transferred twelve times before being used for the 100 day experiment.

It is also not uncommon to find marginal areas of local proliferation which very rapidly alter the whole contour of the culture. Such areas may result from local differences in the rate of multiplication while the whole culture is still active, or from cells continuing to multiply at certain points after proliferation has ceased in adjacent areas

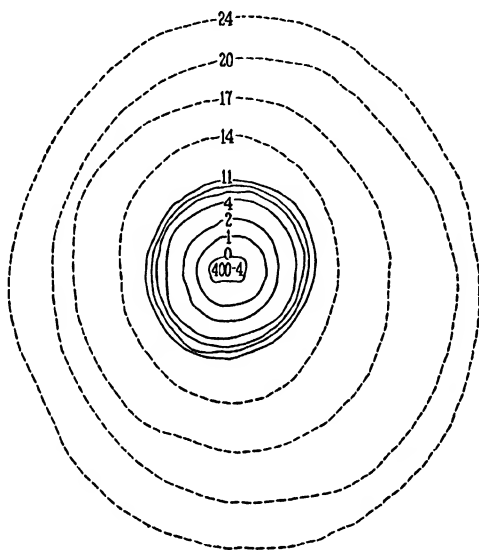


TEXT-FIG. 4. Culture 854-5. Diagrammatic representation of the increase in area of a culture of muscle fibroblasts, in the 12th passage, subjected to the plasma treatment. Note the increasing irregularity in the contour of the culture due to localized cell multiplication.

(Text-fig. 4). In other words, under the conditions of these experiments, it is possible to maintain a cell population comprised of individuals of all ages and in all states of functional activity.

It should be mentioned that the character of the cell growth obtained with plasma is very different from that which is obtained with serum, even when the two are prepared from the same sample of blood. The former brings about cell senescence much less rapidly than the latter. This is not surprising when one considers that serum is far more artificial than plasma and, as such, does not exist in the organism. Serum lacks not only fibrinogen, but possibly also substances of importance in cell nutrition which may be removed together with it.

2. *Transformations from Fibroblasts to Macrophages.*—Whereas certain cell colonies become adjusted to the new environmental conditions without very pronounced changes in the cells themselves, it does not follow that this is always the case. Two sister cultures originating from the same strain and subjected to the same treatment may behave quite differently. To illustrate: After the first series of experiments had been in progress for about 12 days, it was noticed that the

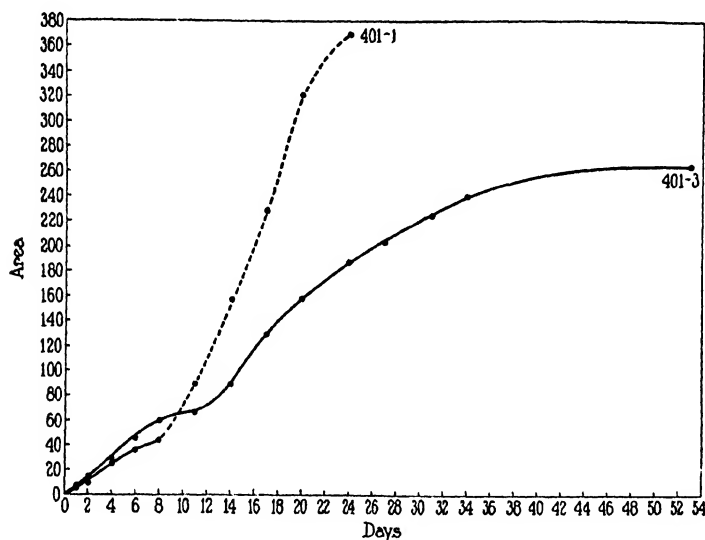


TEXT-FIG. 5. Culture 400-4. Diagrammatic representation of the increase in area of a culture of heart fibroblasts in which transformation to macrophages occurred. The circles represent tracings made from the projected culture. The fibroblasts ceased to increase in area after the 11th day. The broken lines represent areas covered by the macrophages up to the 24th day. (Compare Figs. 8 and 13.)

cells of one of the cultures belonging to a strain of fibroblasts derived from skeletal muscle had very suddenly given rise to a broad band of macrophages (Fig. 14). A few days later, a culture of the same age, but belonging to a strain of heart muscle fibroblasts, behaved similarly (Text-fig. 5; Figs. 8 and 13). Less than 2 weeks later, a third culture showed the same phenomenon. This third culture (Figs. 9-11), which had been treated for 28 days in the flask when the transforma-

tion occurred, had been made from a strain of fibroblasts originating from bone periosteum, a strain that had been carried for twelve passages before the experiment was made. It was obvious, therefore, that the phenomenon was not limited to any one cell type.

After these observations had been made, a new experiment was set up in an endeavor to duplicate as closely as possible every step in the treatment of these cultures in the hope that the changes might recur.



TEXT-FIG. 6. Cultures 401-1 and 401-3. Growth curves of sister cultures of muscle fibroblasts given the same treatment yet placed in separate flasks. Culture 401-1 showed transformation of fibroblasts to macrophages on the 8th day. The broken lines represent the increase in area covered by macrophages. Culture 401-3 did not transform. (Compare Fig. 14.)

This experiment included three cultures of fibroblasts from a heart muscle strain, three from a strain derived from skeletal muscle, and one culture from a strain of fibroblasts isolated from the perichondrium of cartilage. These cultures were made from strains of the same age and had a common past history. After this experiment had been running from 20 to 25 days, the three heart cultures (Figs. 15 and 16) and the three muscle cultures transformed. The culture made from the strain of cartilage fibroblasts, however, did not transform, although

subcultures derived from it were successfully grown on the plasma medium for almost 150 days before the experiment was terminated.

Since but a limited number of the cultures comprising the various experiments showed the phenomenon, it was possible to make a comparative study of the general condition and rate of growth of cultures which had transformed and of those which had not. It was found that those cultures which had not transformed fell into two groups, namely, cultures that responded very favorably to the plasma treatment, as evidenced by the condition of the cells and their rate of growth (Text-figs. 1 and 3; Fig. 12), and cultures in which the cells could not become adjusted to the new medium and were forced to succumb when their residual energy had been utilized. This last condition, which was found but rarely, was restricted to a certain number of cultures which had been derived from the heart strains (Text-fig. 1; Figs. 2 and 3). When transformation did occur, it seemed to take place at some critical period in the life of a culture in which the degeneration process, although evident, was advancing at a relatively gradual rate. It was also found that if two sister cultures belonging to the same experiment had been placed in separate flasks, and one of them transformed whereas the other continued to grow without change, the culture that transformed invariably grew more slowly than the other up to the time when the transformation occurred (Text-fig. 6; Fig. 14).

3. *Growth of Carrel's 19 Year Old Strain of Fibroblasts on a Plasma Medium and Transformation to Macrophages.*—More recently, a slightly modified form of the plasma treatment was used in connection with cultures from the 19 year old strain originally obtained from embryonic heart muscle. This was done in an endeavor to find a means of retaining the material in the laboratory with less effort than is expended by the very frequent transfer of cultures made necessary by the rapid proliferation of cells treated with embryonic tissue juice. The medium differed from that already described only in that about 1.5 per cent of embryonic tissue juice (frozen) was added to the clot in order to bring about rapid coagulation of the plasma. At regular intervals, the medium was washed with Tyrode solution and treated with heparinized plasma, as described above. At the time of writing, cultures have been successfully carried in this manner for over 100 days, during which time they have been transferred to fresh medium eight

times. Taking into consideration the relatively high concentration of embryonic tissue juice necessary for the continued well-being of all strains of fibroblasts derived from embryonic heart,<sup>4</sup> it is safe to assume that the plasma cultures of the 19 year old strain do not owe their present condition to the minute trace of tissue juice added in the preparation of the solid medium. And should its concentration reach a level sufficiently high to be available for cell growth and multiplication, it would be washed out prior to the first treatment with fresh plasma.

61 days after the first of these series of experiments were started, numerous macrophages appeared in a culture which had been treated for 11 days since the previous passage (Figs. 17 and 18). It was a transformation of exactly the same type as that found in the other strains of fibroblasts. In a second series of experiments, a transformation was observed to occur after 68 days of cultivation in the plasma medium. It was apparent, therefore, that the phenomenon was not restricted to those cell strains that had been more recently isolated from the organism.

The possibility of the macrophages being present in the cultures from the moment of their isolation from the tissues of the organism is entirely ruled out, not only by the very nature of the transformation process itself, as will be disclosed later, but also by the conditions of the experiments. Just as plasma is the optimal medium for macrophages, so is a high concentration of embryonic tissue juice toxic to them. A mixed culture of macrophages and fibroblasts, when cultivated for a time on embryonic tissue juice, will soon be found to consist of fibroblasts alone. The macrophages eventually die. Had the tissue been transferred directly from the organism to the plasma medium, it is quite possible that any macrophages present might have survived. In the present experiments, however, this was not the method of procedure. Before being transferred to the plasma diet, the cultures were maintained for an extended period in embryonic tissue juice. Every cell type has its optimal food requirements, and under the conditions of a given nutritional régime there will be a survival of only that cell type able to subsist indefinitely on that particular medium. The recent transformations which have been observed in cultures of the old strain of heart fibroblasts have occurred after a period of culti-

vation of almost 20 years in high concentrations of embryonic tissue juice. The history of this strain alone affords ample proof of the fact that the macrophages could not have been present from the beginning.

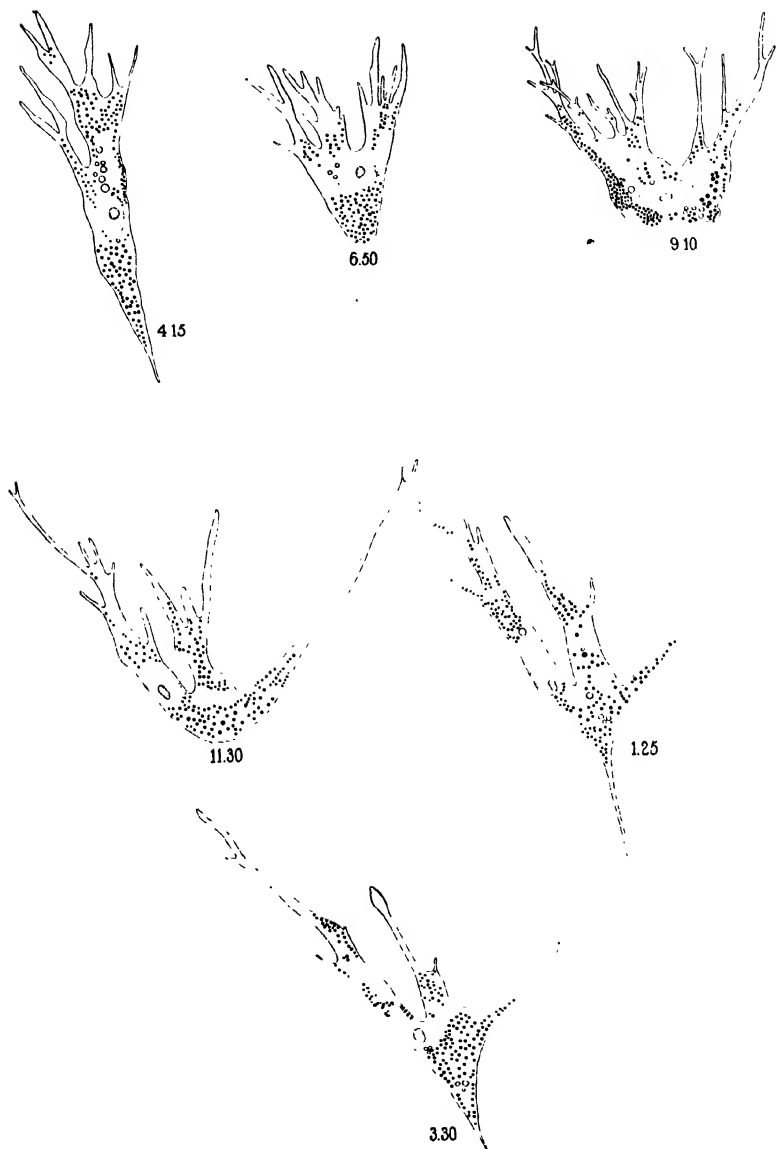
4. *Observations on the Transformation Process.*—The cells that owe their origin to the transformation process (Figs. 10–11, 14–17, 19, 21–22) have been referred to as macrophages because they were identical with the cells which are commonly referred to as macrophages both in form and behavior.<sup>8</sup> They were independent, very active, highly phagocytic, and showed no tendency to form a tissue. They also developed the undulating membrane. Examination of cultures in which the phenomenon was occurring revealed that the macrophages were being budded off from heavily granulated and distended fibroblasts (Figs. 21 and 22).

In order to study the process in detail, it was necessary to select an active fibroblast belonging to a culture in which the macrophages were appearing, and to observe it at 37°C. in the hope that sooner or later something of interest might occur. Although the experiments that were made with the microcinema<sup>9</sup> have not yet happened to show the macrophages actually being budded off from the fibroblasts, it was finally demonstrated with the aid of the camera lucida that this took place by one or more unequal divisions of the mother cell. During these divisions there were no indications of the well known phenomena which accompany mitosis and which make it so easy to detect wherever it occurs. For example, the cells did not round up and there was no “bubbling” of the cytoplasm. The divisions were direct, suggesting amitosis. The camera lucida drawings which showed this have been reproduced (Text-fig. 7). Unfortunately, however, the culture that gave the crucial evidence was contained in a flask of the older type, the walls of which were too thick to permit one to follow with utmost precision the nuclear changes that accompanied the final division of the cell into three separate entities. Each of these was nucleated, and one of them became transformed into a typical macrophage. For this reason, it was necessary to omit the nuclei from the drawings made during the most interesting period. 5 hours after the last drawing

<sup>8</sup> Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, **44**, 285.

<sup>9</sup> The cinematographic experiments were made with the assistance of Mr. Heinz Rosenberger.

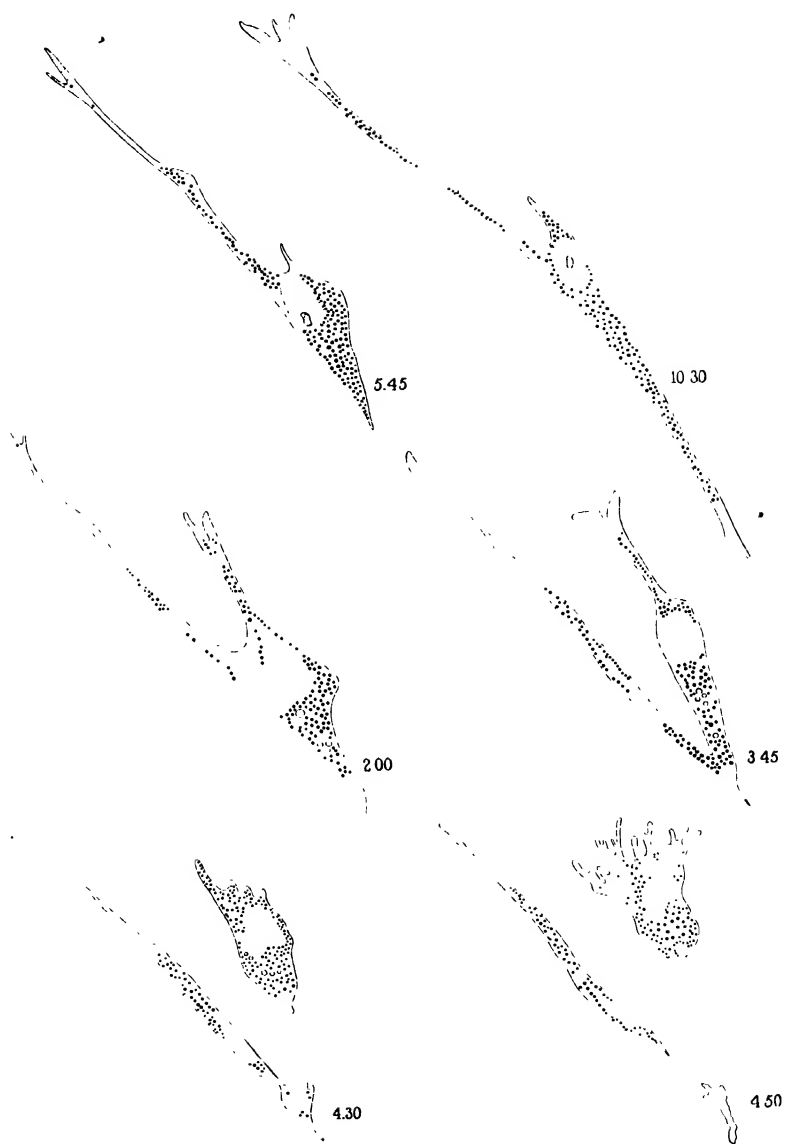




Handlebaum

TEXT-FIG. 7 *a*

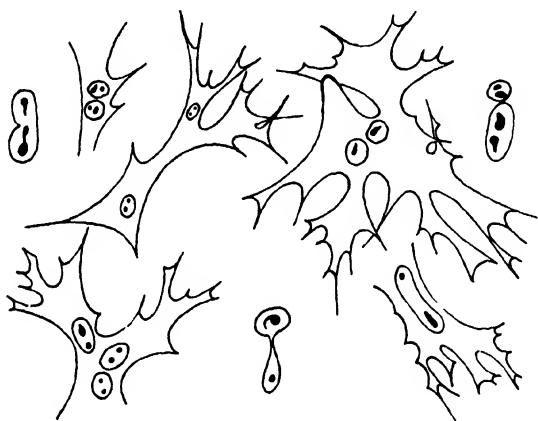
TEXT-FIGS. 7 *a* and 7 *b*. Culture 600-2. Selected camera lucida drawings from a series showing the progressive changes through which a fibroblast passed until its final division into three separate cells, one of which became transformed into a macrophage. Indication is given of the time when each drawing was made.



TEXT-FIG. 7 *b*

was made it was found that several other macrophages had been formed. At this time, nothing remained of the original fibroblast but small remnants in the midst of the macrophage group. These were packed with the larger globules and granules that had been contained in the mother cell.

Previous and subsequent study has shown an almost total absence of karyokinetic figures and other evidences of mitotic divisions in cultures subjected to the conditions of these experiments for long periods of time. Cells containing two or more nuclei are numerous.



TEXT-FIG. 8. Culture 1181. Camera lucida drawings of multinucleate cells and nuclear amitosis from a strain of fibroblasts, in the 19th passage, which had been derived from the perichondrium of cartilage and subjected to the plasma treatment for 178 days. Hematoxylin after Carnoy fixation.  $\times 280$ .

Nuclear amitosis is exceedingly common (Text-fig. 8; Fig. 20). That these multinucleated cells are very active, and are not degenerating cells, is clearly indicated by studies made with the microcinema. In fact, every observation made has been of such a nature as to suggest that cell multiplication in these cultures, as well as the production of macrophages from fibroblasts, may take place amitotically. It is certain that further work with the aid of the microcinema will ultimately furnish more detailed information as to the nature of this type of cell multiplication and the conditions that predispose it.

The production of macrophages, once begun, usually progressed

with amazing rapidity. As a rule, they wandered out into the medium at a uniform rate from the entire periphery of the culture. It was not uncommon for them eventually to cover an area ten or twelve times as great as that finally covered by the colony of fibroblasts from which they were derived (Text-fig. 5; Fig. 8). It was also characteristic of every transformation observed that, as soon as the macrophages were set free, they invariably migrated to a level which was nearer the surface of the clot than the culture itself. The fibroblasts at any point on the margin, and the macrophages that were located directly above them, could be photographed separately simply by making a slight alteration in the focus of the lens (Figs. 9-11, 17-18).

#### DISCUSSION

Cells grown in a medium rich in nitrogenous food substances, as for example, in one containing embryonic tissue juice, do not mature for the reason that they are stimulated to incessant division. The daughter cells arising from one division barely recover before they enter the next. The cells remain forever young. It matters little whether they are originally derived from the embryo or the adult. Sooner or later they all acquire embryonic properties. If, however, the rate of proliferation is reduced to a minimum by the substitution of a pure plasma diet for one in which embryonic tissue juice is present, the cells proliferate at a slower rate and can be left undisturbed for a much longer time before the concentration of metabolic products becomes so great as to inhibit further proliferation. When the plasma treatment is combined with the hanging drop system of cultivation, the volume of medium is so small that cultures have to be transferred every few days in order to maintain a sublethal concentration of catabolites. Thus, the cells are not allowed to remain in any one environment long enough to become accustomed to it. And inasmuch as the increase in tissue volume is negligible in so short a time, the original culture is very soon sacrificed through the mere mechanical procedure of carrying it from passage to passage. This undoubtedly accounts for the failure of every attempt to cultivate normal fibroblasts in serum where the flask technique is not employed.

In the case of the present experiments, in which the plasma treatment is combined with the flask techniques, it has been possible to

keep the cells in good condition over very long periods of time by using large quantities of medium and by periodically washing out the accumulated waste products and treating with fresh plasma. Under these conditions, cultures have been retained in a state of continual growth in the same flask for as long as 100 days. It has been found that, within the confines of a single colony of fibroblasts which have been cultivated for an extended period of time in the plasma medium, the cell variations are numerous. Here we may have, at any one moment, cells of all ages and in all conditions of activity. Within a limited area it is not unusual to find young cells which have recently emerged from division lying side by side with old cells that are still active but have not divided for days, with degenerating cells, and the remnants of cells which have actually succumbed. This heterogeneity may in itself account for much that has been observed in the present experiments. Cells that die and disintegrate must certainly give up substances which very profoundly alter the chemical composition of the medium and thus exert an influence upon the living cells. And the living cells, by creating their own immediate environment, in turn contribute to the environment as a whole. Hence, it is only to be expected that no two cultures would behave similarly under the conditions of the present experiment. Each is an individual with its own peculiar properties.

Reference has already been made<sup>4</sup> to the nutritional differences which exist between various strains of fibroblasts isolated from different tissues of the same organism. The present experiments have shown that, by altering the composition of the pericellular medium, mutants may be produced from the cells of any of these strains. Thus, any of these races, when cultivated for a time on the plasma medium, develop nutritional properties similar to those of macrophages. In fact, it has been observed that certain cells may undergo complete structural transformation to the macrophage type. Under the conditions of these experiments, these changes are irreversible. The macrophages, once formed, will live as long as they are given appropriate treatment, and never completely revert to the fibroblast type. That the cells which result from the transformation process should be identical with macrophages is not remarkable when one considers the food requirements of these cells and the nutritional ré-

gime of the present experiments. Carrel and Ebeling<sup>10</sup> have shown that macrophages are able, not only to utilize plasma and serum substances themselves, but also to elaborate substances which are available for fibroblasts.

Several authors have already reported the occasional occurrence *in vitro* of transformations from fibroblasts to macrophages, although little attempt has been made to define the factors responsible for the changes. In 1926, Carrel and Ebeling<sup>11</sup> reported that twice in the period of 14 years during which the old strain of heart fibroblasts had been cultivated *in vitro*, they had observed a transformation of scattered peripheral cells into macrophages. Still more recently, a similar phenomenon has been reported by Fischer,<sup>12</sup> Ephrussi and Hugues,<sup>13</sup> and W. and M. von Möllendorff.<sup>14</sup>

The reverse process, that is, the transformation of macrophages to fibroblasts, has also been observed to occur. Thus, Carrel and Ebeling<sup>15</sup> reported a transformation of large mononuclears into fibroblastic forms in old cultures in which migration and multiplication had practically ceased. Some years later, Fischer<sup>16</sup> succeeded in obtaining a permanent strain of fibroblasts originally derived from a culture of blood leucocytes. Maximow<sup>17</sup> and Bloom<sup>18</sup> agree that monocytes cultivated *in vitro* may hypertrophy and become transformed, first into macrophages and later into fibroblasts. In this connection, it is of interest to note, however, that it is impossible to distinguish between a monocyte removed from the blood and cultivated for several days in plasma and embryonic tissue juice, and a tissue macrophage. These cells are extremely sensitive to changes in the medium. When, for example, macrophages are cultivated in plasma, they are large

<sup>10</sup> Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, 38, 513.

<sup>11</sup> Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, 44, 261.

<sup>12</sup> Fischer, A., *Arch. exp. Zellforsch.*, 1926, 3, 345.

<sup>13</sup> Ephrussi, B., and Hugues, Y., *Compt. rend. Soc. biol.*, 1930, 105, 697.

<sup>14</sup> von Möllendorff, W., and von Möllendorff, M., *Z. Zellforsch. u. mikr. Anat.*, 1926, 3, 503. von Möllendorff, M., *Z. Zellforsch. u. mikr. Anat.*, 1930, 12, 559.

<sup>15</sup> Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, 36, 365.

<sup>16</sup> Fischer, A., *Compt. rend. Soc. biol.*, 1925, 92, 109.

<sup>17</sup> Maximow, A., *Compt. rend. Soc. biol.*, 1917, 80, 225; *Arch. exp. Zellforsch.*, 1928, 5, 169.

<sup>18</sup> Bloom, W., *Arch. exp. Zellforsch.*, 1928, 5, 269.

and possess a very definite undulating membrane. But when amino peptones are added to the medium, the undulating membrane disappears and the cells become elongated and "snake-like."<sup>19</sup> If a medium is used in which the nitrogenous food materials have been replaced by Tyrode solution, the starved cells become smaller and assume the dimensions and appearance of blood monocytes.<sup>8</sup> These changes, however, are reversible. When brought back to a plasma medium, the cells regain their original features.

Evans and Scott<sup>20</sup> have defended the hypothesis that connective tissue is composed of but two cell types, namely, the phagocytic and the fiber-forming elements (macrophages and fibroblasts). This opinion is based upon the marked difference shown in the response of the two cell types to intravital acid dyes. The present investigation and consideration of the literature show, however, that the cells comprising either of these races may, under certain conditions, appear either as typical fibroblasts or as typical macrophages. The form which the cell takes is but an expression of its physiological state at any one moment. In other words, the fibroblast and the macrophage represent, *in vitro* at any rate, extreme functional states of the same cell type. As macrophages, aside from their typical structural and functional characteristics, the cells display staining reactions with vital dyes which are identical with the reactions of the corresponding cell type freshly removed from the organism.

At this point, one may well ask what criteria exist for the definition of cell types and how one cell type may be distinguished from another. The classical histologists are satisfied to define them on the sole basis of their morphological appearance. That such definitions are inadequate, however, and contribute very little to our knowledge of cells is evidenced not only by the general state of confusion to which they have led, but also by the fact that the workers themselves are never in accord as to the relative values of the various systems of classification which have been so devised. Although it would seem to be self-evident that a cell cannot be conceived of apart from the medium in which it exists, this factor is usually ignored. In order, therefore, to

<sup>19</sup> Carrel, A., *Science*, 1931, 73, 297.

<sup>20</sup> Evans, H. M., and Scott, K. J., *Carnegie Institution of Washington, Pub. No. 273*, 1921, 10, 3.

gain a precise conception of any cell type, it becomes necessary to determine and to specify not only its origin, but also the structural and functional properties which it manifests under given environmental conditions. In other words, descriptions must be based on the actual conditions which exist. For this reason, it has been deemed unnecessary, in connection with the experiments which have been reported, to enter into discussion as to whether the cells studied are or are not identical with the fibroblasts in the tissues of the organism, or what their status may be in terms of the various systems of nomenclature which exist at the present time.

Brief reference should finally be made to the nature of the transformation process itself. In this connection, the findings of the von Möllendorffs<sup>14</sup> are of particular interest. These authors observed frequent transformations of fibroblasts into macrophages, both in inflammation induced by the subcutaneous injection of mice and rabbits with trypan blue, and in cultures of subcutaneous tissue derived from adult rabbits and cultivated over very long periods of time in a medium consisting of a mixture of rabbit plasma and rabbit spleen "extract" containing the same dye substance. Furthermore, although mitoses were occasionally observed in both types of experiments, a high percentage of the cells gave evidence of amitotic division of the nuclei. This led them to believe that the macrophages arose chiefly by amitotic division of the fibroblasts, and that amitosis may be found to occur wherever the metabolism of the cells is disturbed. It has been seen that the present experiments have furnished considerable evidence of this same type of cell multiplication. Whatever its significance may be, it is certain that it should not be overlooked. The mere fact that the idea of amitosis may seem to infringe upon the current theories of cellular heredity is certainly no sound reason either for ignoring its existence or for relegating it to the very convenient category of a degeneration phenomenon.

On the basis of these considerations we may assume, therefore, that the potencies of living cells are far more varied than is generally believed and that the properties which they manifest at any particular moment are functions not only of their inherent capacities, but also of the composition of the environment in which they live and to which they contribute.



## SUMMARY

1. The ability of fibroblasts to mature and to manifest their various potencies in any particular medium is inversely proportional to the growth energy which they exhibit in that medium. Fibroblasts having access to high concentrations of food substances in their environment do not mature, regardless of their origin or the age of the animal from which they were derived. They behave as embryonic cells.

2. Fibroblasts cultivated *in vitro* are potentially able to produce cells with the structural and functional properties commonly attributed to macrophages. This is true regardless of their origin or the length of time which has elapsed since their isolation from the origin.

3. The fibroblast and the macrophage are considered to represent extreme functional and structural variations of the same cell type.

4. The structural and functional characteristics displayed by fibroblasts *in vitro* vary according to their origin and to the changes which take place in the composition of the medium in function of time.

## EXPLANATION OF PLATES

## PLATE 26

FIG. 1. Cultures 1120-1, 1121-3, and 1122-1. Fibroblasts from heart muscle (*H*), skeletal muscle (*M*), and the perichondrium of cartilage (*C*), respectively. These have been cultivated for 22 days in these flasks in a plasma medium, are in their 8th passage, and were made from strains which have received the same treatment from the moment of their isolation from the same chick embryo.  $\times 1$ .

FIG. 2. Culture 396-1. Fibroblasts from heart (*H*), and from the perichondrium of cartilage (*C*) cultivated side by side in the same medium for 77 days; plasma treatment. These cultures are in their 12th passage and were made from sister strains. The fibroblasts from heart have been dead for more than 50 days; the fibroblasts from cartilage are still active. (Compare Text-fig. 1.)  $\times 108$ .

FIG. 3. Culture 396-1. The same cultures after having been treated for 98 days. Note the area of dead heart fibroblasts now invaded by the actively growing cells from the adjacent culture.  $\times 108$ .

FIG. 4. Culture 392-1. Fibroblasts from heart in the 12th passage, photographed after 65 days' cultivation in a plasma medium. This culture was allowed to remain in the flask for 100 days before being transferred. (Compare Text-figs. 2 and 3.)  $\times 8$ .

FIG. 5. Culture 886-3 (from 392-1). Heart fibroblasts from the margin of a 3 day old culture which had been made from transplants taken from the above culture after 100 days' cultivation in the first flask. When photographed, these

cells were in their 13th passage and had been cultivated for 103 days in the plasma medium.  $\times 115$ .

FIG. 6. Culture 1177. Peripheral cells from an 8 day old culture of fibroblasts from heart belonging to a strain originally derived from 392-1. When photographed, these cells were in their 18th passage and had been cultivated in the plasma medium for 183 days.  $\times 108$ .

FIG. 7. Culture 112-2. Serial section of culture of fibroblasts from cartilage, in the 13th passage, cultivated for 44 days in the plasma medium. Stained with hematoxylin after fixation with formol-Zenker without acetic.  $\times 272$ .

#### PLATE 27

FIG. 8. Culture 400-4. Transformed culture of fibroblasts from heart, in the 5th passage, cultivated for 15 days in the plasma medium. Note the broad band of macrophages. Fig. 13 shows a photograph of this culture made 3 days earlier. (Compare Text-fig. 5.)  $\times 9$ .

FIG. 9. Culture 395-1. Degenerated cells from the margin of a transformed culture of fibroblasts from the periosteum of bone. This culture was in its 12th passage and had received the plasma treatment for 29 days.  $\times 111$ .

FIG. 10. Culture 395-1. Lower magnification of margin of the same culture, showing both macrophages and the shadows of the fibroblasts (out of focus).  $\times 41$ .

FIG. 11. Culture 395-1. Macrophages from the same culture.  $\times 111$ .

FIG. 12. Culture 399-1. Active cells from the margin of a culture of fibroblasts from the periosteum of bone, belonging to the same series of experiments. These cells are also in their 12th passage and have received the plasma treatment for 42 days without transformation having occurred.  $\times 111$ .

FIG. 13. Culture 400-4. Fibroblasts from heart, in the 5th passage, cultivated for 12 days in the plasma medium. 3 days later this culture transformed. (Compare Fig. 8.)  $\times 9$ .

FIG. 14. Culture 401-1. Macrophages from the margin of a transformed culture of fibroblasts from muscle in the 5th passage. This culture had received the plasma treatment for 12 days before transformation occurred.  $\times 41$ .

#### PLATE 28

FIG. 15. Culture 599-1. Transformed culture of fibroblasts from heart, in the 5th passage, after having received the plasma treatment for 21 days.  $\times 21$ .

FIG. 16. Culture 599-1. Higher magnification of macrophages from the margin of the same culture.  $\times 108$ .

FIG. 17. Culture 8000 C1. Macrophages from a transformed culture of the 19 year old strain of fibroblasts from heart, after 61 days of cultivation in a plasma medium.  $\times 216$ .

FIG. 18. Culture 8000 C1. Atypical fibroblasts from the margin of the same culture. For the purpose of reproduction, it was necessary to accentuate the large upper cell.  $\times 216$ .

FIG. 19. Culture 768 (from 600-2). Macrophages from a culture of fibro-

blasts from muscle which had transformed in the previous (5th) passage. At the time of transformation, the culture belonged to the series of experiments which included Culture 599-1 (Figs. 15 and 16). Both strains were of the same age and had a common past history.  $\times 216$ .

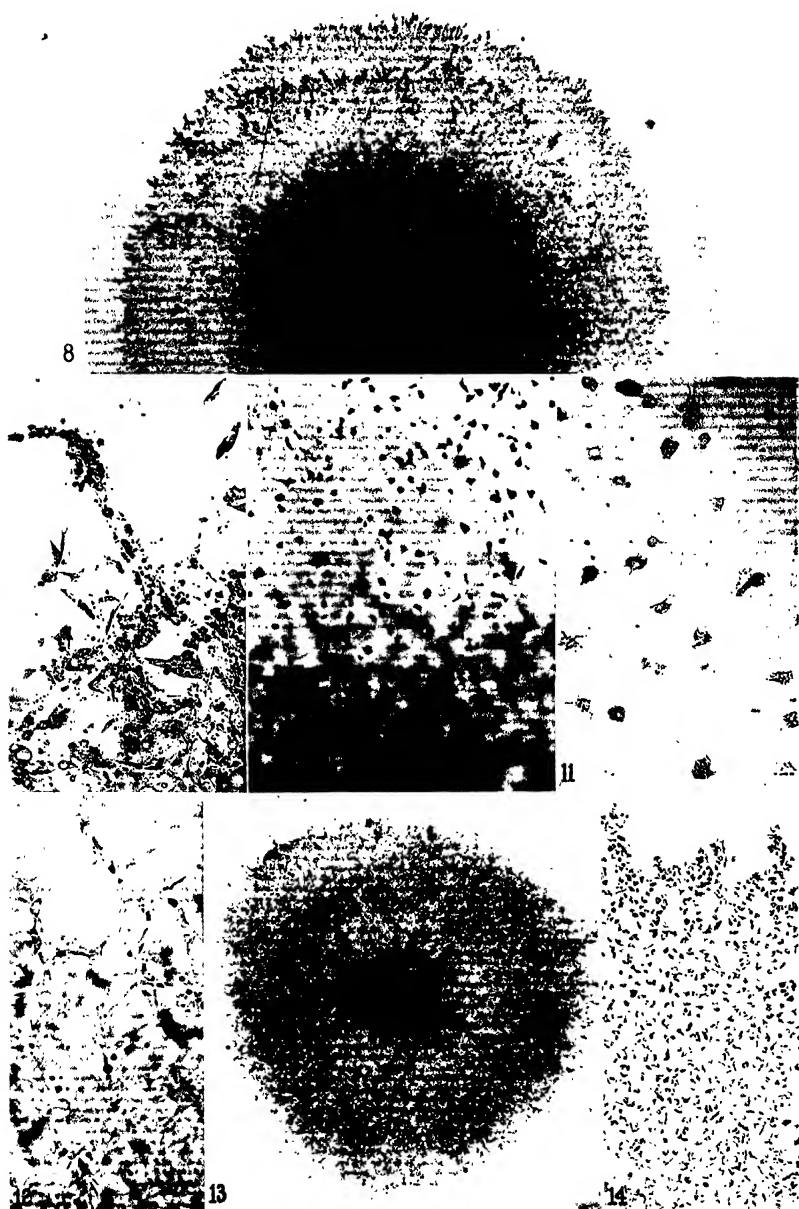
FIG. 20. Culture 1272-1. Cells from a strain of fibroblasts from muscle, in the 13th passage, which have been cultivated for 26 days on lipoid-free serum protein, and stained supravitaly with neutral red. Note the binucleated cell and the absence of fat globules.  $\times 216$ .

FIG. 21. Culture 600-2. Two macrophages and an atypical fibroblast from the culture of fibroblasts from muscle referred to above (Fig. 19). Note the distended, irregular, and heavily granulated appearance of the fibroblast-like cell.  $\times 376$ .

FIG. 22. Culture 600-2. This photograph shows a number of macrophages, one of which is in the process of segregating itself from the remnant of a fibroblast. (Compare Text-fig. 7.)  $\times 376$ .

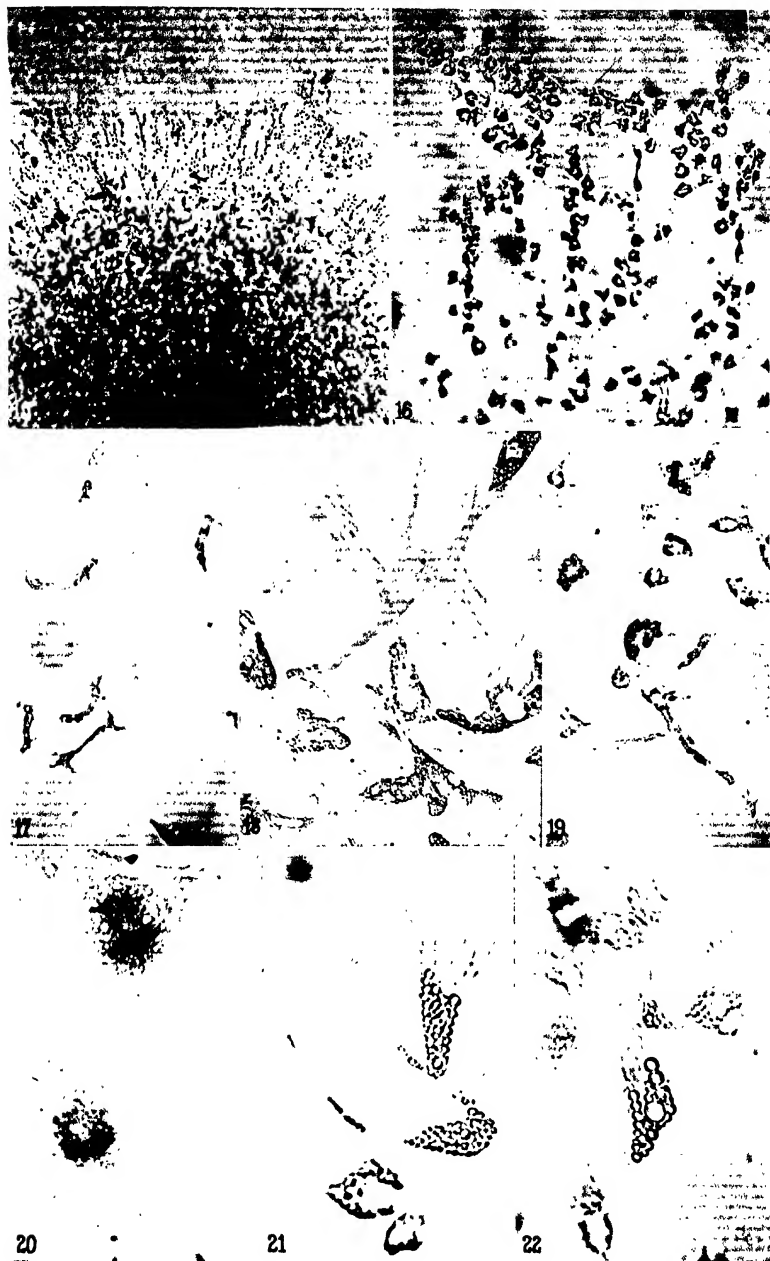






Parker, Races constituting common fibroblasts, D





(Parker. Races constituting common fibroblasts. 1)





## THE BLOOD PIGMENTS OF URECHIS CAUPO

By J. P. BAUMBERGER AND L. MICHAELIS

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The echiurid *Urechis caupo* was discovered by Fisher and MacGinitie and is an abundant inhabitant of the Monterey Bay in California.<sup>1</sup> One of its interesting features is its richness in hemoglobin. This has been the subject of an extended study by Redfield and Florkin.<sup>2</sup> It is a peculiarity of this invertebrate that its hemoglobin is contained within the blood cells and none in the blood fluid. Another localization of the hemoglobin is the muscles, which are not vascularized but contain all hemoglobin within the muscle cells; and, furthermore, the dorsal nerve chord appears red with hemoglobin. There are, however, several other particular aspects with respect to the blood pigment which are to be presented in this paper. In part, they are concerned with changes, probably according to the age or to the seasons, which could not be fully studied during one season. The description of these changes will be presented as they appeared to be and may be subject to modifications as further studies may be extended over a longer period of time.

The animals at our disposal varied in length, in the contracted state, from 3 to 10 inches. Accordingly, the blood content of the body cavity varied from 10 to 30 cubic centimeters. The color of the blood varies, from the purest oxyhemoglobin-red to the darkest brown-black or a black like chinese ink, even after complete saturation with oxygen. This variation of the blood is a very striking feature and obviously has a definite physiological significance.

Red blood was encountered in some few of the smallest individuals, and in some of the very largest sex-mature females. The majority of the individuals, of medium size, contained brown or brown-black

<sup>1</sup> Fisher, W. K., and MacGinitie, G. E., (1928), *Ann. and Mag. Nat. Hist., Ser.* 10, vol. 1, p. 199 and p. 204.

<sup>2</sup> Redfield, E., and Florkin, M., 1931. *Biol. Bull.*, 61: 185.

blood. The blackest blood ever encountered was that of a very large sex-mature male. The cause of the difference in color is revealed by a microscopic examination. The red color of the blood is due to hemoglobin homogeneously distributed within the blood cells. Whenever the color is brown, besides this hemoglobin there is another, granular, pigment of brown color within the cells which will be proved to be hematin. The description of the changes in these pigments may be presented according to ideas developed during a study of two months. This may not be sufficient to make sure of all details, and the whole picture may be liable to some modifications upon more extended studies.

We start from a pure red blood in a young animal, recalling the fact that not every small animal of our material contained the blood in the red condition. In such an animal, the blood cells are spherical, about 10–15  $\mu$  in diameter. The protoplasm is diffusely yellowish-green with hemoglobin and, besides, rather tightly packed with colorless granula of regular spherical shape, of a rather high refractory index,—though not so high as that of fat drops,—and about 1  $\mu$  in size. No nucleus is visible in the fresh preparation but a nucleus becomes visible after fixing and staining (fixed in acetone and stained with safranin). The nucleus is small, in the centre of the cell, and contains a distinct nucleolus. Besides these cells, there is another kind, usually somewhat smaller, much less numerous, containing yolk-yellow droplets of a considerable size which often are conglomerated into a mulberry-like packet.

When the blood becomes brown, the granula of the hemoglobin-containing cells are no longer colorless but are stained with a brown pigment. The granula, then, are no longer quite uniform in size and spherical in shape, but somewhat more irregular. The size of the cell is the same as in the red blood of young worms. This aspect was most common among our material.

Now we come to the large sex-mature worms. Here a difference arises according to the sex. One feature is common for both sexes. The corpuscles become larger, up to 35  $\mu$  in diameter, and more variable in size. In the males, the brown pigment no longer stays exclusively within the granula, but is more homogeneously scattered over the cell so that the hemoglobin color is overshadowed and can be detected

only by the spectroscope. The granula at the same time undergo a disintegration. They swell and have indistinct contours, being, as it were, dispersed into a turbid mass without definite structure. At the same time very small, spherical, quite black pigment granula, very dense in structure, and not very numerous, are formed within the cell. We do not know whether the development will go beyond this stage, but it appears as though all hematin would gradually disappear and in part be converted into the dense black pigment.

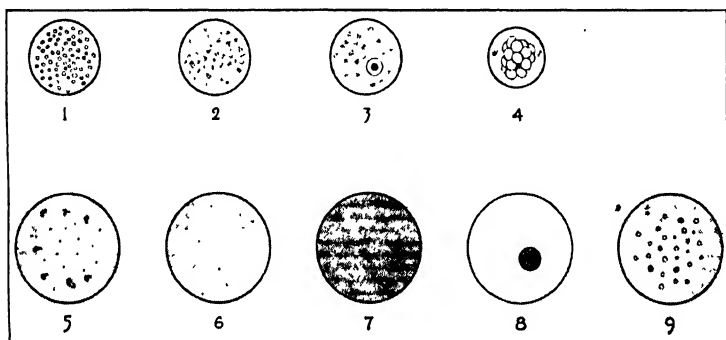


FIG. 1. Blood cell with regular, colorless granula of a relatively high refractory power. Hemoglobin is diffusely dissolved in the protoplasma, not in the granula. Fresh preparation.

FIG. 2. Blood cell with hematin-stained granule. Granula brown, protoplasma yellowish green with hemoglobin. Fresh preparation.

FIG. 3. The same, dry smear, fixed with acetone and stained with safranin. Nucleus with nucleolus.

FIG. 4. Smaller cell with yolk-yellow droplets, containing sometimes also a few hematin granula. Fresh preparation.

FIG. 5. Larger blood cell of a sex-mature female, with only a few hematin granula. The whole protoplasma is diffusely yellowish-green with hemoglobin, with no distinct structure. Fresh preparation.

FIG. 6. Larger blood of a sex-mature female, yellowish-green with hemoglobin, without any hematin, very fine colorless granula of low refractory power. Fresh preparation.

FIG. 7. The same, without any distinct granular structure, pure hemoglobin shade in the whole protoplasma. Fresh preparation.

FIG. 8. The same, dry smear, fixed with acetone and stained with safranin, showing the nucleus.

FIG. 9. Blood cell of an old male, black pigment besides colorless granula. The protoplasma is diffusely light brown. Fresh preparation.

The disintegration of the brown granula takes place in the females also; but it does not lead to the formation of black granula within the blood cells. Rather is the blood cell gradually deprived of any pigment except for the hemoglobin. Instead, a pigment is formed within the eggs, and there can be little doubt that the brown blood pigment is the source of the black egg pigment.

The egg is a very large cell of almost the same aspect as that of *Asterias*, also with respect to the size and shape of the nucleus. After insemination, the nucleus disappears and the polar bodies are formed. In the protoplasm of the egg a very fine dust of pigment granula is scattered. The number of these granula is not very large so that the eggs show macroscopically only a very slight yellowish-grey shade. These pigment granula are in part black, in part somewhat more dark red. The black pigment has the same shade as the one in the erythrocytes of the male, the difference being only that the black granula in the eggs are usually smaller than those in the male erythrocytes. Upon confronting the fact that the black pigment is met, in the males, only within the blood cells and never in the sperm, and in the females only in the eggs and never in the blood cells, the interpretation seems unavoidable that the brown pigment is the mother material for the black one and is utilized for the eggs in the female, but remains in the blood cells of males.

It is likely that the brown pigment (which will be identified with hematin) is converted, in part, into the black granular pigment, and also in part into hemoglobin again. This latter conclusion is suggestive because the sex-mature females with purely red blood have blood cells of a much larger size than younger animals and yet these cells certainly do not contain the hemoglobin in a lower concentration.

The blood cells can be hemolyzed by a copious amount of distilled water, or in the undiluted blood, by some drops of ether, or better, by gently shaking with a drop of octyl alcohol. The granula described above will float isolated in a preparation of the laked blood. The colorless granula remain as individuals, very often also the brown granula, though these may also be disintegrated to finer pigment granula of yellow-brown color. All transitions can thus be observed from colorless granula to partially and completely stained granula.

The chemical behavior of the hemoglobin has been fully described

by Redfield and Florkin. It agrees in all its reactions and in all optic properties with mammalian hemoglobin. It can be separated from the brown pigment simply by centrifuging the blood hemolyzed with a drop of octyl alcohol. The brown pigment is entirely insoluble and forms the main part of the cake-like sediment, whereas the hemoglobin is dissolved in the supernatant liquid. The brown pigment can be extracted from the cake-like sediment in the following way: The cake is first extracted with acetone (or ether). A yolk-yellow pigment is herewith extracted which is present either in the blood fluid or in the yolk-yellow cells described above. When this extraction is complete, another extraction is performed with acetone (or ether) containing acid (glacial acetic acid or some drops of strong HCl). Hereupon the brown pigment goes into solution and reveals the characteristic bands of acid hematin. When this solution is reduced, either by shaking with solid sodium hydrosulfite, or with platinum asbestos and hydrogen, and pyridine is added, the characteristic spectrum of pyridine-hemochromogen arises with its very distinct two bands even in highest dilution. The brown pigment has herewith identified itself with hematin. The pyridine-hemochromogen prepared from the hemoglobin, by treatment with acid acetone, reduction and addition of pyridine, is spectroscopically identical with the one prepared in the same way from the hematin granula. Both from the hemoglobin and from the brown granular pigment Teichmann's crystals could be obtained.

It may be alluring to venture an interpretation of the physiological significance of the changes occurring in the blood of this animal. We prefer, however, to refrain from such an interpretation until experiments of a more physiological nature are available.



## AN EQUATION FOR ELECTROLYTIC CONDUCTANCE

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(Received for publication, December 11, 1931)

Debye and Hückel<sup>1</sup> have proposed a theory for the effect of the ionic atmosphere on the mobilities of ions. According to that theory the decrease in equivalent conductance should be proportional to the square root of the concentration, a relation which was first found empirically by Kohlrausch. The theoretical calculations for the decrease of ionic mobility with increasing concentration consider two effects. In the first place, when an ion moves through a solution under the influence of an applied electric field, it tends to disturb the surrounding ionic atmosphere, which then exerts an opposing electric force. In the second place, the ions comprising the ionic atmosphere produce a counter current of solvent which also retards the motion of the central ion.

However, Onsager<sup>2</sup> has pointed out that in calculating the first or so-called ionic effect, Debye and Hückel neglected to take into account the influence of the Brownian movement of the ions, and also used a treatment which is valid only if the negative and positive ions have equal mobilities. Furthermore, he has shown that the second or electrophoretic effect could be calculated in a manner which does not involve the ionic radii. Onsager's treatment likewise confirmed the square-root relationship between the equivalent conductance decrease and concentration, and, what is very important, the proportionality factor in his theoretical expression can be readily calculated from well-known constants.

Although Onsager's equation has been shown to be in close agreement with measurements at very low concentrations, it is strictly valid only as a limiting expression. This is so because in the derivation of

<sup>1</sup> Debye and Hückel, *Physik. Z.*, **24**, 305 (1923).

<sup>2</sup> Onsager, *ibid.*, **27**, 388 (1926); **28**, 277 (1927).



the simple equation, only the first approximations were retained in the mathematical treatment, and also the simple physical picture assumed in the derivation may be inadequate for finite concentrations.

Numerous empirical equations have been proposed to correlate conductance measurements with concentration, and to serve for extrapolations of equivalent conductances at infinite dilution. An empirical formula suitable for the latter purpose should, if the recent theoretical deductions are correct, reduce in the limit of zero concentration to the Onsager equation.

Recently Ferguson and Vogel<sup>3</sup> and Lattey<sup>4</sup> have proposed conductance formulas, which, however, do not appear to be satisfactory for the purpose of extrapolation. These formulas have been criticized by Davies and others.<sup>5</sup>

In this paper an equation is suggested which is an empirical extension of Onsager's limiting expression, containing only one additional (empirical) constant. It agrees with measurements up to a concentration to about one-tenth normal, and yields Onsager's equation in the limit.

### *The Onsager Equation*

Onsager's equation for the mobility of an ion is

$$l = l^0 - \left[ \frac{(0.9838) (10^6)}{(DT)^{3/2}} w l^0 + \frac{28.95 Z}{(DT)^{1/2} \eta} \right] \sqrt{(Z_1 + Z_2)C} \quad (1)$$

$l^0$  is the mobility at infinite dilution

$D$  is the dielectric constant of the solvent

$T$  is the absolute temperature

$\eta$  is the viscosity of the solvent

$Z$  is the charge carried by the ion (absolute value)

$C$  is the equivalent concentration

$Z_1$  and  $Z_2$  are the charges carried by the anions and cations

$l_1^0$  and  $l_2^0$  are the mobilities at infinite dilution of anions and cations

$$w = Z_1 Z_2 \frac{2q}{1 + \sqrt{q}}; \quad q = \frac{Z_1 Z_2 (l_1^0 + l_2^0)}{(Z_1 + Z_2)(Z_2 l_1^0 + Z_1 l_2^0)}$$

<sup>3</sup> Ferguson and Vogel, *Phil. Mag.*, **50**, 971 (1925); **4**, 1, 233, 300 (1927); **5**, 199 (1928).

<sup>4</sup> Lattey, *ibid.*, **4**, 831 (1927).

<sup>5</sup> Davies, "The Conductivity of Solutions," John Wiley and Sons, Inc., New York, 1930, pp. 81-85, 113; *Trans. Faraday Soc.*, **27**, 597 (1931).

The first term in the brackets takes account of the ionic effect, and the second term of the electrophoretic effect, both of which decrease the mobility of the ions. For the equivalent conductance,  $\Lambda$ , of a uni-univalent electrolyte the expression

$$\Lambda = \Lambda_0 - \left[ \frac{5.78 \times 10^6}{(DT)^{3/2}} \Lambda_0 + \frac{58.0}{(DT)^{1/2} \eta} \right] \sqrt{2C} \quad (2)$$

in which  $\Lambda_0$  is the equivalent conductance at zero concentration, is thus obtained.

Since, as has already been noted, all higher terms have been neglected in the derivation of this equation, it is, strictly speaking, a limiting formula. The effect of retaining higher terms, however, would be such as to decrease the rate at which  $\Lambda$  falls off with concentration according to the above equation. This is in accord with the facts for strong electrolytes, and Debye and Hückel and also Onsager have used an additional empirical term, linear with concentration, for purposes of extrapolating for  $\Lambda_0$  from measurements of dilute solutions. However, this extension is valid only to concentrations of several thousandths normal. If, on the other hand, the electrolyte had a tendency to associate as the concentration is increased, the observed deviations from the values predicted by Equation 2 would be in the opposite direction.

For convenience let us rewrite Equation 2 in simpler form

$$\Lambda = \Lambda_0 - (\alpha\Lambda_0 + \beta) \sqrt{C} \quad (3)$$

where  $(\alpha\Lambda_0 + \beta) = A$  is the limiting theoretical slope. Solving for  $\Lambda_0$  we obtain

$$\Lambda_0 = \frac{\Lambda + \beta \sqrt{C}}{1 - \alpha \sqrt{C}} \quad (4)$$

which is, of course, only another way of writing the Onsager equation.

For solutions in water at 25°  $\alpha$  is 0.2274 and  $\beta$  is 59.79, using the recent data of Drake, Pierce and Dow<sup>6</sup> and of Wyman<sup>7</sup> for the dielectric

<sup>6</sup> Drake, Pierce and Dow, *Phys. Rev.*, **35**, 613 (1930).

<sup>7</sup> Wyman, *ibid.*, **35**, 623 (1930).

constant of water, and the values given in the "International Critical Tables" for the other constants. At  $18^\circ$   $\alpha$  is 0.225 and  $\beta$  is 50.3.

### *An Extension of the Onsager Equation*

In examining the conductivity of numerous strong uni-univalent electrolytes it was found that values of  $\Lambda_0$  as calculated from Equation

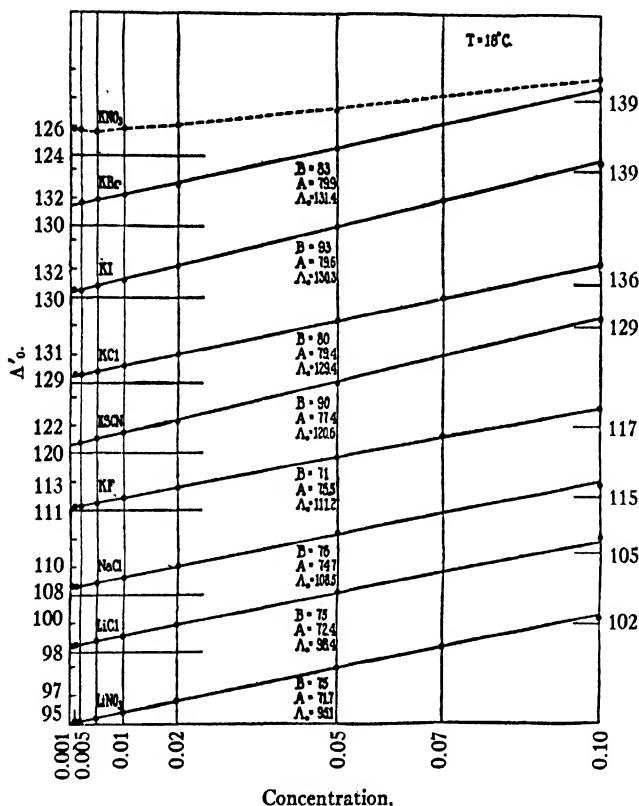


FIG. 1

4 are not constant over any appreciable concentration range. Thus the data do not follow the limiting Onsager equation. However, I have observed that these computed values, which will be designated by  $\Lambda'_0$ , plotted against the first power of the concentration, usually give straight lines up to about 0.1 normal. On this basis the correct  $\Lambda_0$

value for each electrolyte is the intercept of the  $\Lambda'_0$  vs.  $C$  line at  $C = 0$ . Figure 1 shows plots of  $\Lambda'_0$  vs.  $C$  for aqueous solutions of a number of strong electrolytes at  $18^\circ$ . The data are taken from the "International Critical Tables" and from the results of Kohlrausch and his

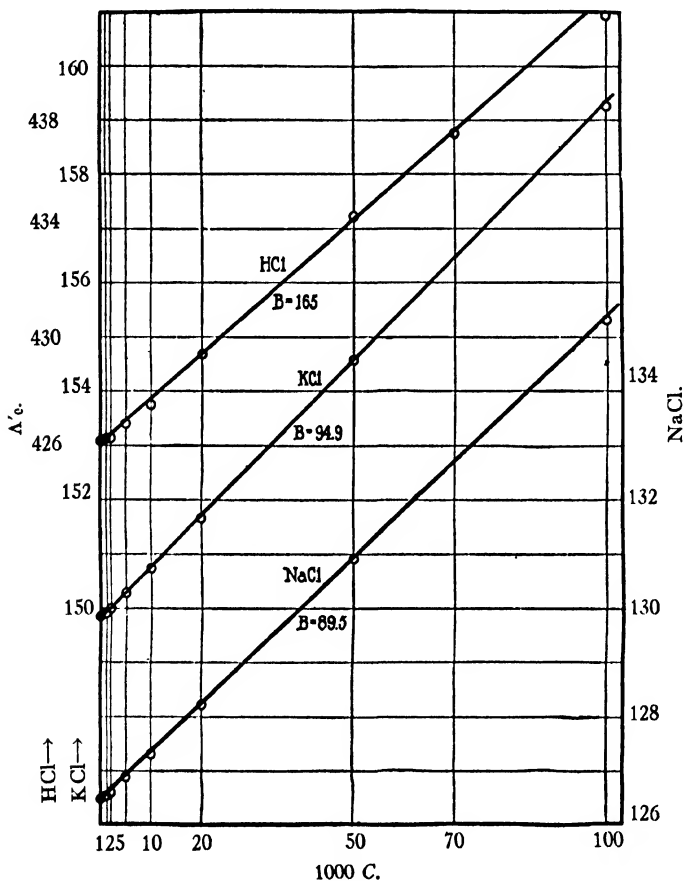


FIG. 2

co-workers. Figure 2 shows the same relationship for solutions of several electrolytes at  $25^\circ$ , the data for which have been obtained in this Laboratory with an improved conductivity technique.<sup>8</sup> If the

<sup>8</sup> Shedlovsky, *THIS JOURNAL*, **54**, 1411 (1932).

slope of these lines is represented by  $B$ , we have the following conductance equation

$$\Lambda_0 = \frac{\Lambda + \beta \sqrt{C}}{1 - \alpha \sqrt{C}} - BC \quad (5)$$

instead of the limiting equation (4).

It is an interesting fact that for many electrolytes the slopes  $B$  of these lines have values not far from the magnitude of  $A = \alpha \Lambda_0 + \beta$ , in Onsager's limiting equation (Equation 3).

An examination of Equation 5 shows that it approaches the limiting equation of Onsager asymptotically.  $\alpha$  and  $\beta$  are the theoretical constants of Onsager's equation. It should be noted that Equation 5 is not equivalent to the addition of a linear term in the direct expression for equivalent conductance; *i. e.*,  $\Lambda = \Lambda_0 - (\alpha \Lambda_0 + \beta) \sqrt{C} + BC$ , which, as mentioned above, has been used by Onsager for extrapolating  $\Lambda_0$  from measurements at concentrations below 0.01 normal. The expression just given rearranges to

$$\Lambda_0 = \frac{\Lambda + BC + \beta \sqrt{C}}{1 - \alpha \sqrt{C}}$$

instead of Equation 5, in which the term  $BC$  is added to  $\Lambda_0$  instead of to  $\Lambda$ .

The substances which conform to Equation 5 (Figs. 1 and 2) belong to the class of strong electrolytes, which according to the modern theory of solutions are considered to be essentially entirely ionized. It is found that for these cases  $A$  is generally somewhat smaller than  $B$  but within 15% of it.

However, in the plot for potassium nitrate, represented by the dotted curve in Fig. 1, the linear relationship between  $\Lambda'_0$  and  $C$  of Equation 5 does not hold. Similar behavior is exhibited by the chlorates and iodates, and by thallium salts. We may tentatively assume that Equation 5, with  $B$  near to or larger than  $A$ , represents the behavior of strong uni-univalent electrolytes. On this basis the substances resembling potassium nitrate behave in a manner which would result from association. Equation 5 may also be useful as a guide in calculating degrees of dissociation.

It is often more convenient to deal with an expression explicit in  $\Lambda$ . For this purpose Equation 5 can be rearranged in the form

$$\Lambda = \Lambda_0 - A \sqrt{C} + BC(1 - \alpha \sqrt{C}) \quad (6)$$

where  $A = \alpha \Lambda_0 + \beta$ .

TABLE I

*Equivalent Conductance (18°). Comparison of Observed and Computed Values*

C	LiNO <sub>3</sub>		LiCl		NaCl		KF	
	Calcd.	Obs.	Calcd.	Obs.	Calcd.	Obs.	Calcd.	Obs.
0	95.1		98.4		108.5		111.2	
0.001	92.9	92.9	96.2	96.2	106.2	106.3	108.9	108.9
.002	92.0	92.0	95.3	95.3	105.3	105.3	108.0	107.9
.005	90.4	90.3	93.7	93.6	103.6	103.5	106.2	106.2
.01	88.6	88.6	91.9	91.8	101.7	101.7	104.4	104.3
.02	86.5	86.4	89.6	89.6	99.4	99.4	102.0	101.9
.05	82.6	82.7	85.9	85.9	95.4	95.5	97.7	97.7
.07	81.1	81.1	...	...	...	...	95.9	96.0
.10	79.4	79.2	82.7	82.8	92.0	91.8	94.0	94.0
A	71.7		72.4		74.7		75.5	
B	75		77		76		71	
C	KSCN		KCl		KI		KBr	
	Calcd.	Obs.	Calcd.	Obs.	Calcd.	Obs.	Calcd.	Obs.
0	120.6		129.4		130.3		131.4	
0.001	118.3	118.4	127.0	127.1	127.9	128.0	129.0	129.1
.002	117.3	117.4	126.0	126.0	126.9	127.0	128.0	128.0
.005	115.5	115.5	124.2	124.2	125.1	125.1	126.2	126.1
.01	113.7	113.7	122.2	122.2	123.3	123.2	124.2	124.1
.02	111.4	111.3	119.8	119.7	120.8	120.8	121.7	121.6
.05	107.6	107.5	115.5	115.5	116.9	117.0	117.5	117.5
.07	106.0	106.1	113.6	113.6	115.4	115.5	...	...
.10	104.5	104.5	111.7	111.8	113.8	113.8	113.9	114.0
A	77.4		79.4		79.6		79.9	
B	90		80		93		83	

Table I shows a comparison between the observed values of the equivalent conductance,  $\Lambda$ , and those calculated from Equation 6 for the solutions of salts at 18° corresponding to the linear plots in Fig. 1. The agreement is within the limits of experimental error for these data.

Table VI of the following paper shows the same comparison for our own measurements at 25° on solutions of potassium chloride, sodium chloride and hydrochloric acid.<sup>8</sup>

Equation 6 is consistent with the theoretical prediction of Onsager<sup>2</sup> that the first higher order term in his derivation, were it not neglected, would be linear in concentration and opposite in sign to the square root term.

Lattey's equation,<sup>4</sup> which is  $\Lambda = \Lambda_0 - (A'\sqrt{C})/(1 + B'\sqrt{C})$  (in which  $\Lambda_0$ ,  $A'$  and  $B'$  are adjustable constants), when applied to data on strong uni-univalent electrolytes does not necessarily approach Onsager's limiting equation. In fact Lattey's  $A'$  is usually found to be appreciably greater than the theoretical limiting slope,  $A = \alpha \Lambda_0 + \beta$ . Also, as an extrapolation formula it gives values for  $\Lambda_0$  which appear to be too high, if Onsager's expression is valid for sufficiently low concentrations.

The equation proposed in this paper is an extension of Onsager's theoretical equation. It has been found to be valid up to a concentration of about tenth normal. As an extrapolation formula for most strong uni-univalent electrolytes it gives values of  $\Lambda_0$  from data at concentrations which can be measured readily. These  $\Lambda_0$  values will agree with values obtained from Onsager's equation applied to measurements on sufficiently dilute solutions.<sup>9</sup>

#### SUMMARY

An equation is proposed for the relation between the equivalent conductance of strong uni-univalent electrolytes and the concentration. It has been found to agree with measurements up to about 0.1 normal in aqueous solutions. Besides theoretically calculable constants and the limiting conductance,  $\Lambda_0$ , it contains only one additional constant, and reduces to the limiting Onsager equation.

<sup>9</sup> The author has made some preliminary tests of Equation 5 on strong uni-univalent electrolytes. It appears to hold in a perfectly analogous manner for these cases if the concentrations are expressed as "ionic strengths." The application of the equation to this and higher valence types will be communicated at a later date to THIS JOURNAL.

## THE ELECTROLYTIC CONDUCTIVITY OF SOME UNI- UNIVALENT ELECTROLYTES IN WATER AT 25°

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### INTRODUCTION

It has appeared important to obtain accurate values for the electrolytic conductance of solutions of a number of electrolytes. Besides providing a test for the conductivity relationships predicted by the modern theory of ionic solutions, such measurements make possible the calculation of the degrees of dissociation of certain weak electrolytes, as is shown in another paper.<sup>1</sup> Also, when combined with determinations of transference numbers, they give values for individual ionic mobilities. This will be the subject of a communication from this Laboratory in the near future.

With the alternating current bridge method, which has been almost universally used since Kohlrausch for measuring electrolytic conductance, a sensitivity of one part in 100,000 in the resistance readings can be readily attained. Due to various sources of error, some of which will be mentioned below, the accuracy of such measurements may not reach one part in 1000. In fact there are comparatively few measurements available for which a consistent accuracy of more than several hundredths of one per cent. has actually been attained.

In this paper new measurements are reported on the conductivity of several uni-univalent electrolytes in water at 25°, covering a concentration range from about  $2 \times 10^{-5}$  to 0.1 normal. They were obtained with an apparatus and a technique designed to give a consistent accuracy of one or two hundredths of a per cent. for the relative conductance values.

<sup>1</sup> MacInnes and Shedlovsky, THIS JOURNAL, 54, 1429 (1932).



*Experimental*

*Bridge, etc.*—The alternating current bridge, oscillator, and detector used in this research have been described by the author in a previous paper.<sup>2</sup> With this apparatus it is possible to make measurements at several frequencies. This is important because most of the electrical errors change with the frequency, providing a useful criterion for detecting such errors.

It was shown in the earlier paper that the sources of error in the bridge (apart from the calibration of the rheostat) amounted to less than 0.002% when the direct current values of metallic resistances were compared with the corresponding a. c. measurements up to 3000 cycles. The coils in the rheostat were frequently calibrated against several Bureau of Standards standard resistances which had been certified by the Bureau of Standards, and the relative accuracy of these calibrations is a few hundredths of an ohm for the individual coils. The coils of 1000 ohms or over were immersed in a thermostat to avoid temperature fluctuations.

In measuring very dilute solutions, when cell resistances are greater than 10,000 ohms, I have found it expedient to shunt the cell with a 10,000-ohm resistance, free from reactance errors at the frequencies used. By measuring first this shunt resistance, and then the parallel combination of the cell and the shunt, the resistance of the cell is readily calculated from the relation  $\rho = SR/(S-R)$  in which  $\rho$  is the desired resistance of the cell,  $S$  the resistance measurement of the shunt and  $R$  the resistance measurement of the parallel combination. Of course, the use of such a shunt method results in a loss of precision. However,  $S$  and  $R$  are both known to 0.1 ohm, since the bridge easily has that sensitivity when measuring resistances of the order of 10,000 ohms, and the calibrations of the individual coils are accurate to 0.05 ohm, as mentioned above. Consequently, an accuracy of 0.01% is retained when  $S-R$ , the determining factor, amounts to 1000 ohms, which corresponds to a resistance of about 90,000 ohms in the cells. That this method has the precision just stated was shown by making check measurements with shunts differing in resistance by 2000 ohms.

<sup>2</sup> Shedlovsky, *THIS JOURNAL*, **52**, 1793 (1930).

*Conductivity Cells.*—Jones and Bollinger<sup>3</sup> have shown in a recent paper that many cells which are commonly used in careful conductivity work suffer from a source of error inherent in the design. In such cells, in which the filling tubes are relatively close to the electrode leads, disturbing parasitic currents can flow through capacity-resistance paths, and these can produce apparent variations in cell constants. A variation of cell constant at high resistances had been observed by Parker,<sup>4</sup> who attributed the effect to adsorption. Jones and Bollinger have, however, definitely proved that this so-called "Parker effect" disappears when the filling tubes and electrode leads in the cells are spaced in a manner which avoids appreciable stray currents. Their new cells, which are designed with this in view, give measurements which are constant at several frequencies, indicating that this type of error has been eliminated. The measurements I have made on solutions of concentrations greater than 0.005 normal were carried out in cells of the type recommended by these authors. These cells were made from Jena 16<sup>III</sup> glass and the electrodes were lightly platinized.

With very dilute solutions it is desirable to use a cell of relatively large volume, so that increasing concentrations can be built up and successively measured without risk of contamination from atmospheric or other impurities. Cells of this type have been described by Weiland,<sup>5</sup> Kraus and Parker,<sup>6</sup> Hartley and Barrett,<sup>7</sup> and have been generally used in recent work on dilute solutions. These workers used cells consisting essentially of a flask with a pair of dipping electrodes as is represented diagrammatically in Fig. 1 (a). However, as Jones and Bollinger have pointed out, dipping electrodes may also lead to errors in conductivity measurements. The following brief analysis will make clear the reason for such errors and the direction of their effect on the measurements.

With the cell indicated in Fig. 1 (a) the total current measured in the bridge consists, when polarization is negligible, not only of the current

<sup>3</sup> Jones and Bollinger, *THIS JOURNAL*, **53**, 411 (1931).

<sup>4</sup> Parker, *ibid.*, **45**, 1366, 2017 (1923).

<sup>5</sup> Weiland, *ibid.*, **40**, 133 (1918).

<sup>6</sup> Kraus and Parker, *ibid.*, **44**, 2429 (1922).

<sup>7</sup> Hartley and Barrett, *J. Chem. Soc.*, **103**, 786 (1913).

flowing directly between the electrodes through the solution and a capacity current between the electrodes (including the leads), but there is also a parasitic current which flows from the electrode leads by electrostatic capacity to the solution and then through it. This latter current is neither constant nor directly proportional to the resistance of the solution, and thus tends to produce apparent variations in the cell constant.

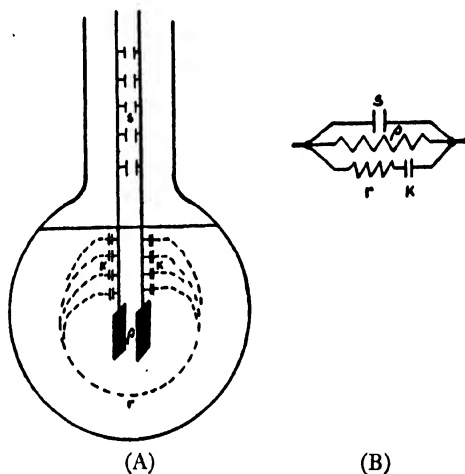


FIG. 1

The electrical circuit for the cell in Fig. 1 (a) is shown sufficiently closely for our purpose by the accompanying diagram Fig. 1 (b). Since this circuit is balanced in the bridge by a resistance and a capacity in parallel, we have the following condition to balance<sup>8</sup>

$$\frac{1}{R} + j\omega c = \frac{1}{\rho} + j\omega S + \frac{1}{r - j/\omega\kappa} \quad (1)$$

in which  $\rho$  is the resistance of the solution between the electrodes,  $S$  the electrostatic capacity between the electrodes including their leads,  $r$  and  $\kappa$  are the resistance and capacity components of the disturbing parasitic current,  $R$  is the resistance reading and  $c$  the capacity reading in the bridge,  $\omega$  the angular frequency, and  $j$  the reactance operator.

<sup>8</sup> See, for instance, Hague, "Alternating Current Bridge Methods," p. 71.

Solving Equation 1 for  $1/R$  we obtain

$$\frac{1}{R} = \frac{1}{\rho} + \frac{1}{r + (1/\omega^2\kappa^2\rho)} \quad (2)$$

Since  $r$  depends on the resistance of the solution, it is directly proportional to  $\rho$ , that is,  $r = a\rho$ , where  $a$  is a constant. Thus

$$\frac{1}{R} = \frac{1}{\rho} \left( 1 + \frac{1}{a + (1/\omega^2\kappa^2a\rho^2)} \right) \quad (3)$$

which, on expansion, neglecting higher terms, gives

$$\frac{1}{R} = \frac{1}{\rho} \left( 1 + \frac{1}{a} - \frac{1}{\omega^2\kappa^2a^2\rho^2} \right) \quad (4)$$

The term  $1/\omega^2\kappa^2a^2\rho^2$  which we shall call  $d$  varies with the square of the frequency and the square of the resistance of the solution.

It is evident from this treatment that a parasitic current resulting from a path consisting of a capacity in series with a resistance such as  $r$  and  $\kappa$  in Fig. 1 will produce errors in the conductivity measurements if balanced, as in the usual bridge, with a resistance and a pure capacity. However, no error results from a shunting current in the cell through a pure capacity such as  $S$  in the same figure. Since the correction terms in Equations 3 and 4, arising from the disturbing parasitic current, contain factors not readily determined, it is better to design cells so as to eliminate such currents, rather than to attempt to correct for them.

Equation 4 accounts for the observation that measurements made with dipping electrodes vary with the frequency. Furthermore, if the cell is calibrated with a solution having a resistance  $\rho_0$  for which the term  $1/\omega^2\kappa^2a^2\rho^2 = d_0$ , measurements at higher resistances or higher frequencies will result in conductance values which are apparently too high, since  $d$  will decrease progressively with increasing resistance or frequency. And, by similar reasoning, the apparent conductances will be too low for resistances less than  $\rho_0$ .

To avoid this error I have constructed cells in which disturbing parasitic currents are not present. Such a cell is shown in Fig. 2 (a). In this design, which includes Jones and Bollinger's recommendations, the electrodes are taken out of the flask, the leads to the electrodes are

separated from each other and the space between is filled not with the solution but with the oil in the thermostat. Consequently the disturbing parasitic currents are made negligible. The lead M, nearest to the flask, is connected to the terminal of the bridge which is maintained at earth potential, so that capacity effects to earth from this relatively large surface are avoided. It has been found on repeated tests that conductance values obtained with this cell are independent of frequency, between 1000 and 3000 cycles.

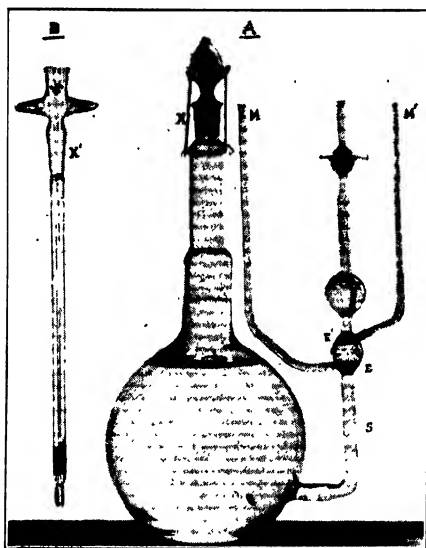


FIG. 2

As an experimental proof of the electrical error due to dipping electrodes Fig. 2 (b) measurements with them have been compared with corresponding ones made in the cell shown in Fig. 2 (a), using the same solutions. The dipping electrodes were firmly set into the flask of the cell shown in Fig. 2 (a) through the ground joint x-x'. The results of the comparison are shown graphically in Fig. 3. In this plot the ordinates correspond to the ratio of values obtained with the dipping electrodes Rd and those obtained with our cell R. The abscissas correspond to the resistance as measured by the immersed electrodes. Since the readings obtained with the cell shown in Fig. 2 (a) are inde-

pendent of frequency, they are considered to be correct. On this basis the curves in Fig. 3 represent the apparent variations of the cell constant of the dipping electrode with resistance at the frequency indicated. In accord with Equation 4 the variation increases with the resistance and the frequency.

Some further essential details of the design of the cell shown in Fig. 2 (a) are as follows. The flask, of transparent quartz, has a capacity of 1 liter. The cell proper is made of Jena 16<sup>III</sup> glass connecting to the flask through a graded seal S. The electrodes E, E' are truncated hollow cones of platinum foil, the outer surfaces of which are completely sealed to the glass. They are platinized only to a dull gray

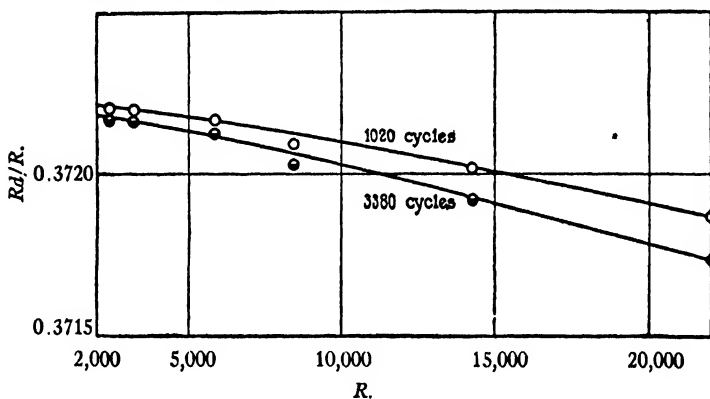


FIG. 3

color, which is sufficient to avoid polarization at the concentrations used in this cell. Electrical contact is made with the electrodes through the mercury cups M, M', which are amply spaced to avoid parasitic shunting currents. To eliminate temperature errors the leads from the cups extend into two short test-tubes of mercury supported in the thermostat. Two additional leads connect these tubes to the bridge. Three cells of this design of somewhat different dimensions have been used in measuring dilute solutions of potassium chloride and sodium chloride. The conductance values obtained in all three were entirely consistent with each other.

*Temperature Control.*—The temperature of the solutions measured was controlled by immersion of the cells in an electrically operated

thermostat filled with a light mineral oil. Jones and Josephs<sup>9</sup> pointed out that appreciable errors may result from the use of water in the thermostat in measurements with alternating currents. The regulator consists of a hollow-walled cylindrical steel tube filled with about 300 cc. of mercury, connecting to a glass capillary in which is set the regulating needle. By use of a radio tube arrangement, similar to that described by Beaver and Beaver,<sup>10</sup> sparking at the mercury surface is entirely avoided. The heater consists of bare manganin wire wound non-inductively on an insulated frame around the regulator. The temperature of the thermostat, which remained constant to  $\pm 0.001^\circ$ , was set accurately at  $25^\circ$  ( $\pm 0.001^\circ$ ) with the aid of a carefully calibrated platinum resistance thermometer, which serves as a temperature standard in this Laboratory. It is of the strain-free type, with a resistance of about 25 ohms and is provided with a special thermostated Mueller thermometer bridge and a sensitive galvanometer.

### *Purification of Materials*

*Conductivity Water.*—The conductivity water was obtained by redistilling distilled water from alkaline potassium permanganate in a still similar to the one described by Kraus and Dexter.<sup>11</sup> The precautions suggested by these authors were carefully observed. In this apparatus the steam from the evaporator passes through a settling chamber in which traces of spray are separated from the vapor. This vapor then passes to a condenser where it is partially condensed. The large volume of uncondensed vapor carries with it most of the carbon dioxide and other gaseous impurities. The condensed water was collected out of direct contact with air in a 15-liter "Vitreosil" quartz flask.<sup>12</sup> This flask was fitted with an air-tight block tin cover carrying block tin pipes for collecting and withdrawing the water. The conductivity water in this container was protected from atmospheric impurities by a set of "guard" bottles, containing sulfuric acid and strong potassium hydroxide solution, followed by a long tube filled with soda lime. The specific conductance of the water was between 0.1 and  $0.4 \times 10^{-6}$  mhos.

*Sodium and Potassium Chlorides.*—The best obtainable c. p. salts were crystallized once by absorbing pure hydrochloric acid gas in a hot saturated solution which was gradually cooled to  $0^\circ$ . After centrifuging, the product was recrystal-

<sup>9</sup> Jones and Josephs, *THIS JOURNAL*, **50**, 1065 (1928).

<sup>10</sup> Beaver and Beaver, *Ind. Eng. Chem.*, **15**, 359 (1923).

<sup>11</sup> Kraus and Dexter, *THIS JOURNAL*, **44**, 2469 (1922).

<sup>12</sup> In the measurements on hydrochloric acid (dilute) the conductivity water was collected directly from the still into the cell. Its conductivity was always less than  $2 \times 10^{-7}$ .

lized from conductivity water, again centrifuged, and dried in a clean electric oven. Two samples of potassium chloride were prepared by four crystallizations from conductivity water, but no difference was observed between the conductance of solutions made with them and with the samples prepared as above. The purified salts were fused in platinum under vacuum in an electric furnace. When potassium chloride and particularly sodium chloride is fused in air, the resulting product is slightly alkaline, as can be seen from a pink fringe around a crystal placed in a few drops of distilled water containing a trace of phenolphthalein. However, fusing the salts in vacuum avoids this difficulty, probably by removing traces of water quickly enough to prevent appreciable hydrolysis at the high temperatures.

*Potassium Nitrate and Silver Nitrate.*—The c. p. salts<sup>13</sup> were recrystallized several times from conductivity water, centrifuged, dried and fused as above. The fused salts gave negative tests with phenolphthalein and with acidified potassium iodide and starch solution, showing that no loss or decomposition of nitrate took place.

A "Richard's bottling apparatus,"<sup>14</sup> was used in handling the fused salts, since they are more or less hygroscopic. With this apparatus<sup>15</sup> it is possible to fuse salts in a controlled atmosphere, and to transfer them into glass-stoppered weighing bottles where they may be kept without risk of contamination from the air.

*Hydrochloric Acid.*—Constant boiling hydrochloric acid was prepared from c. p. acid in the manner described by MacInnes and Dole.<sup>16</sup> The barometric pressure corresponding to the fraction of acid collected was carefully noted, and the concentration of the product was thus determined from the data of Foulk and Hollingsworth.<sup>17</sup> Analyses were also made by differential potentiometric titrations (through a sodium hydroxide solution) against benzoic acid and potassium hydrogen phthalate with the procedure described by MacInnes and Cowperthwaite.<sup>18</sup> The three methods agreed well within 0.01%. Although the distilled acid, which was stored in small glass-stoppered Pyrex flasks, showed no appreciable change in concentration on standing for several weeks, fresh samples were taken for making up the solutions used in this work.

*Determination of Cell Constants.*—The "Jones and Bollinger"<sup>19</sup> cell for moderately concentrated solutions (cell B) was calibrated with a 0.1 "demal" solution of potassium chloride, containing 7.47896 g. of potassium chloride and 1000 g. of water (weighed in air). The specific conductance of this solution according to Parker and Parker<sup>19</sup> is 0.012852<sub>4</sub> at 25°. This value has been taken as the basis for all the conductance measurements reported in the present paper.

<sup>13</sup> A pure sample of silver nitrate was kindly supplied by Dr. Vinal of the Bureau of Standards. This sample was recrystallized once from conductivity water.

<sup>14</sup> T. W. Richards and H. G. Parker, *Proc. Am. Acad. Arts Sci.*, **32**, 59 (1896).

<sup>15</sup> This excellent apparatus, which is essential for accurate work with hygroscopic materials, has not come into as general use as might be desired.

<sup>16</sup> MacInnes and Dole, *THIS JOURNAL*, **51**, 1119 (1929).

<sup>17</sup> Foulk and Hollingsworth, *ibid.*, **45**, 1220 (1923).

<sup>18</sup> MacInnes and Cowperthwaite, *ibid.*, **53**, 555 (1931).

<sup>19</sup> Parker and Parker, *ibid.*, **46**, 332 (1924).



Another "Jones and Bollinger" cell (C) had a resistance of less than 600 ohms when filled with 0.1 "dema" potassium chloride solution. But since it is best to measure cell resistances of over 1000 ohms to retain the full sensitivity of the bridge, the cell C was calibrated by intercomparison with cell B. The ratio between measurements in these two cells remained constant to 0.01% over a wide range of resistances with solutions of different electrolytes, *i.e.*, no "Parker effect" was observed. The cell constants showed only slight variations with time over a period of two years. In that time the constant of cell B,  $K_B$ , varied between 24.706 and 24.712; and that of cell C,  $K_C$ , varied between 7.6342 and 7.6357. In computing the conductance values obtained in these cells, which were calibrated at intervals of two or three months, the most recent calibration was always used.

The cell constants of the "flask" cells Fig. 2 (a) which were used in measuring the very dilute solutions, were determined at the end of each run in these cells by intercomparison with cell C. Also for these cells no "Parker effect" was observed. For three cells of this type the constants were (1) 0.70652, (2) 0.51247, (3) 0.58915; cell (1) was used in series a, b, d, e on potassium chloride, and series a, b on sodium chloride; cell (2) was used in series f on potassium chloride and series c, d, e, f on sodium chloride; cell (3) was used in all the other dilute series.

*Preparation of Solutions.*—Solutions at round concentrations of 0.1, 0.05, 0.02, 0.01, 0.005 and in some cases 0.07 normal at 25° (gram moles per liter of solution at 25°) were made up by weight, using the density data given in the "International Critical Tables." The glassware was cleaned with hot dichromate-sulfuric acid solution, thoroughly rinsed with distilled water, steamed out, and dried with air which had been passed through towers of fused calcium chloride followed by a long cotton filter. After determining the dry weight of the flasks, they were again rinsed with several samples of conductivity water before being used for making up the solutions.

*Weighing.*—Three balances were used for weighing in this research. A large Christian Becker chainomatic balance with a capacity of 10 kilos and an average sensitivity of 0.5 mg. was used in weighing water for the solutions. A Christian Becker analytical balance, with a sensitivity of 0.05–0.1 mg. was used for weighings corresponding to loads between 10 and 150 g. A Kuhlman microbalance with a sensitivity of 0.001 mg. was used for weighing small quantities of solutes. All weighings were made by the tare and substitution method, and were corrected to vacuum. The weights for the large and micro balances were carefully calibrated to the same basis against a standard set which was certified by the Bureau of Standards.

### Procedure

*Measurements above 0.005 Normal.*—The conductivities of solutions above 0.005 normal, which were made up to round concentrations of 0.005, 0.01, 0.02, 0.05, 0.07 and 0.10 normal,<sup>20</sup> were determined in cells B and C. These cells were

<sup>20</sup> Check series of solutions were kindly prepared by Mr. Donald Belcher of this Laboratory.

periodically cleaned with dichromate-sulfuric acid mixture, followed by very thorough rinsing with distilled water, and finally with conductivity water, with which they were also kept filled when not in use. Occasionally, when it was suspected that the cells had been contaminated with grease, they were cleaned with alcohol and ether before receiving the usual cleaning treatment.

The procedure was to rinse the cell used in a particular measurement with six or eight portions of the solution, and then to let the cell stand, filled with solution, for fifteen to thirty minutes. The cell was then refilled with solution and immersed in the thermostat, where it attained thermal equilibrium within about fifteen minutes, after which its resistance remained constant. Refilling the cell and repeating the measurement usually gave the same resistance value, but several fillings of the cell with check measurements were always made.

For transferring the solution from the storage flask into the cells used for the more concentrated solutions, the ground-glass stopper of the flask was replaced by a two-holed rubber stopper into which were fitted a short glass tube and a long U-shaped glass tube, the latter reaching to the bottom of the flask. The tubes were, of course, previously cleaned and dried. The outside end of the U-tube was connected to the cell, and the solution was forced into it with a sufficient pressure of purified air.

The solvent correction for the solutions measured in this manner is relatively small. It was determined by finding the conductivity of a sample of the water used in making up the solution in a cell similar in design to cells B and C, but having large electrodes and a cell constant of about 0.037. The conductance of the water used for these solutions, since it was not completely protected from the carbon dioxide in the atmosphere, was between  $0.5$  and  $0.8 \times 10^{-6}$  mhos, usually about  $0.6 \times 10^{-6}$ . However, this value corresponds to about only 0.1% of the conductivity of a 0.005 normal solution for the electrolytes measured.

*Measurements of Very Dilute Solutions.*—The conductivities of the most dilute solutions were measured in the "flask" cell Fig. 2(a). The cell, which was thoroughly cleaned in the manner described below, was partially filled with about 950 cc. of fresh conductivity water. The solvent was then swept out for several minutes with purified nitrogen entering through the stopcock and escaping through the neck of the flask. (The stopcock and ground-glass stopper in the "flask" cell were lubricated with a trace of thick, purified vaseline. This was prepared by distilling off the more volatile components of white vaseline under a vacuum, and the viscous residue was extracted repeatedly with hot conductivity water.) The cell with the water was then weighed, and the quantity of solvent was determined by subtracting the weight of the empty dry cell. After introducing mercury into the tubes leading to the cell electrodes, the cell was placed in the thermostat and connected to a source of purified nitrogen, etc., in the manner shown in Fig. 4. The mercury cup  $E_1$ , which is nearest to the flask and makes contact with the lower electrode in the cell proper (W), was connected to the bridge terminal at the junction of the rheostat and detector. Owing to the relatively large surface of the flask, it has an appreciable capacity to earth. However, by connecting  $E_1$  to the terminal which

is at earth potential when the bridge is balanced, no capacity current to earth is likely from this source. The ground-glass stopper (S) was held in place with a rubber band. By means of the three-way stopcock A, the cell could be connected either to a source of purified nitrogen under a pressure, regulated by the reduction valve V, or to the atmosphere through the guard train D. With this arrangement (T open and A as in Fig. 4), the valve V was set so that a slight pressure was established in the flask (F). This pressure was regulated to force solution into the cell proper (W) past the electrodes, half filling the bulb (B) when the stopcock A was subsequently turned so as to connect T to the atmosphere (through D). Thus the cell W could be alternately filled and emptied by turning A to the proper position. With A set as in Fig. 4, the liquid in the flask could be stirred with purified nitrogen by lifting the stopper S slightly.

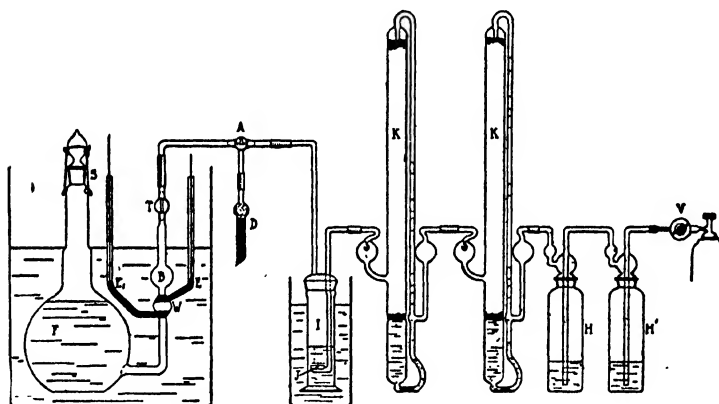


FIG. 4

The purification train for the nitrogen consists of two gas scrubbing bottles containing sulfuric acid (H, H'), two scrubbing towers (K) of the type recommended by Kraus and Parker,<sup>21</sup> filled with strong potassium hydroxide solution, and finally the glass filter bottle (I) which contains conductivity water and is immersed in the thermostat. In this bottle, the gas passed through the sintered glass membrane (J) in fine bubbles, and is thus saturated with water vapor at the temperature of the thermostat.

The guard train (D) consists of a tube of sulfuric acid, and a long soda lime tube. The purpose of this train is to prevent atmospheric impurities from diffusing into the cell when it is opened to the air by the stopcock A.

After the conductivity water in the "flask" cell had reached thermal equilibrium in the thermostat, the cell proper (W) was filled, as described above, by manipulating the stopcock (A) with the stopcock (T) open. Then (T) was closed and the

<sup>21</sup> Kraus and Parker, *THIS JOURNAL*, **44**, 2432 (1922).

resistance was measured. This procedure was repeated a number of times to make sure that a reliable, constant value was obtained.

Then, with the ground-glass stopper (S) removed and a slow stream of nitrogen passing through the flask, an increment of solute was quickly added and the stopper replaced. This was added in the form of salt fused in tiny platinum cups, which were dropped into the flask, or in the form of solution of known concentration which was added from a weight buret. Two weight burets were used, one having a capacity of 1.5 cc. and the other 75 cc. Both burets were made from Pyrex glass. In the case of the platinum cups, containing 20 to 90 mg. of fused salt, and the micro buret, containing a maximum of 1.5 cc. of solution, the required weighings were made on the micro balance to 0.001 or 0.002 mg. When the larger buret was used, at least 5 g. of solution was added, and the weighings were made on the analytical balance to 0.1 mg. The small platinum cups were handled with platinum-tipped tongs, the micro buret with clean, oil-free chamois, and the larger buret with clean silk gloves. For the most dilute solutions the quantities of solid required were too small to weigh accurately. In such cases small quantities of solution of known concentration were added from the micro weight buret. However, most of the values given for potassium chloride, sodium chloride and potassium nitrate were obtained with the use of the platinum cups. These weighed about 0.25 g., their exact weight being determined for each run. They were readily cleaned by rinsing in a stream of distilled water and heating to incandescence over a Bunsen burner until no color was imparted to the flame. The small amounts of salt were fused in the cups in the manner already described. After the addition of an increment of solute, the stopcock T was closed, the flask cell was disconnected, removed from the thermostat, and thoroughly shaken until all the added solute was well distributed. Then the flask was replaced in the thermostat, reconnected, and, by manipulating (A), (T) and (S), the cell proper was filled and emptied numerous times, and the solution stirred with gas. After thermal equilibrium had again been established, the solution was measured, as described above, with several refillings of the cell proper (W). This procedure was repeated until several concentrations had been built up and measured. In the case of some very low concentrations, especially with hydrochloric acid, it was found that the measured resistance for a particular filling of C changed with time. It is supposed that this effect was due to adsorption or slight contamination from the Jena glass of which the cell proper is constructed (the flask itself being of quartz). However, since the relative volume of the cell proper is small, this effect on the solution as a whole was probably negligible. In any case, reproducible values were always obtained by taking the readings immediately on filling (W), or by taking readings over a period of time and extrapolating to zero time. The level of the solution in the bulb (B) or in the flask had no detectable effect on the measurements.

After the conductance of the last solution had been determined in this apparatus, it was again measured in cell C. Using the result of this measurement, it was possible to check the constant of cell (W) at the end of each series of determinations.

Before starting a new run, the flask cell was completely cleaned inside and outside. Most of the oil adhering to the outside of the cell was removed with warm soap solution. The mercury cups were cleaned out with nitric acid. All grease was finally removed with ether, the flask and cell were completely filled with warm sulfuric acid-dichromate mixture and allowed to stand overnight. It was then thoroughly rinsed with distilled water by letting at least 5 liters flow through it, entering at the stopcock and leaving through the neck of the flask. The quartz

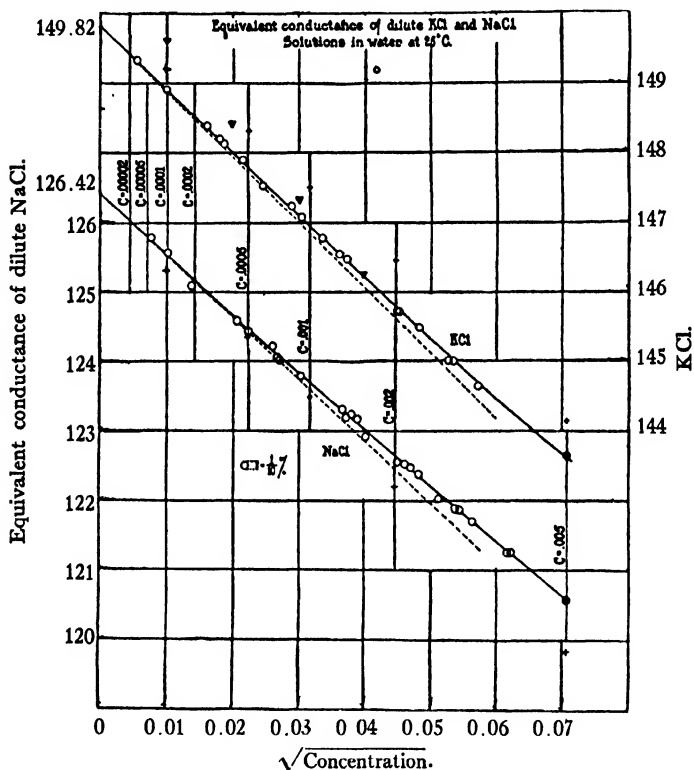


FIG. 5.—O, Shedlovsky; ∇, Grindley and Davies; +, Jeffery and Vogel

flask was then further cleaned with steam, and rinsed with several portions of conductivity water. The outside of the flask and also the mercury cups were dried and the apparatus was ready for the next run.

## RESULTS

All the conductance values here presented are on the basis of a specific conductance of 0.012852<sub>4</sub> for a 1/10 "dema" potassium

chloride solution at 25°. <sup>19</sup> Such a solution contains 7.47896 g. of potassium chloride in 1000 g. of water (weights in air *vs.* brass).

TABLE I  
*Equivalent Conductances of Potassium Chloride at 25°*

Series	$C \times 10^4$	$\Lambda$	Series	$C \times 10^4$	$\Lambda$
f	0.32576	149.33	d	11.321	146.76
f	1.0445	148.91	e	14.080	146.46
f	2.6570	148.38	d	15.959	146.26
e	3.3277	148.19	b	20.291	145.72
b	3.5217	148.12	e	20.568	145.71
c	4.6948	147.89	d	23.379	145.48
a	6.0895	147.52	a	27.848	145.00
e	8.4200	147.23	e	28.777	144.99
c	9.2856	147.07	b	32.827	144.64

$$\Lambda_0 = 149.82$$

TABLE II  
*Equivalent Conductances of Sodium Chloride at 25°*

Series	$C \times 10^4$	$\Lambda$	Series	$C \times 10^4$	$\Lambda$
e	0.59441	125.79	f	15.306	123.17
e	1.1283	125.57	d	16.271	122.91
d	1.8985	125.09	b	20.348	122.54
d	4.2677	124.58	c	21.253	122.51
f	5.0056	124.44	f	22.275	122.47
c	6.7898	124.21	a	23.388	122.37
a	6.9196	124.21	b	26.538	122.02
d	7.1846	124.05	c	28.988	121.88
b	7.3843	124.02	—	29.806	121.87
f	9.2243	123.78	a	31.862	121.70
c	13.448	123.31	c	37.367	121.26
b	13.868	123.19	a	38.776	121.26
a	14.466	123.24			

$$\Lambda_0 = 126.42$$

The equivalent conductances are defined as  $\Lambda = 1000 L/C$ ,  $L$  being the measured specific conductance, and  $C$  the concentration in equivalents per liter of solution at 25°. The molecular weight values

TABLE III  
Equivalent Conductances of Hydrochloric Acid at 25°

Series	$C \times 10^4$	$\Lambda$	Series	$C \times 10^4$	$\Lambda$
a	0.28408	425.01	a	7.5404	421.66
c	0.81181	424.75	b	15.768	419.88
b	1.7743	423.82	a	18.766	419.64*
a	3.1863	423.43	c	25.614	418.32
c	3.4227	423.22	b	29.943	417.98
b	5.9146	422.42			

$$\Lambda_0 = 426.04$$

TABLE IV  
Equivalent Conductances of Potassium Nitrate at 25°

Series	$C \times 10^4$	$\Lambda$	Series	$C \times 10^4$	$\Lambda$
a	0.69820	144.17	b	12.119	141.53
a	1.7613	143.62	a	16.468	140.91
a	3.8888	142.98	b	24.219	140.17
b	5.8651	142.61	b	36.724	139.08
a	8.6853	141.97			

$$\Lambda_0 = 144.92$$

TABLE V  
Equivalent Conductances of Silver Nitrate at 25°

Series	$C \times 10^4$	$\Lambda$	Series	$C \times 10^4$	$\Lambda$
a	0.27575	132.91	a	7.5380	130.82
b	0.72453	132.64	b	10.026	130.45
a	1.0710	132.48	b	12.297	130.10
a	3.5387	131.58	a	14.530	129.86
b	4.6704	131.46	b	29.054	128.44

$$\Lambda_0 = 133.32$$

given in the "International Critical Tables" have been used throughout. Except in the case of the hydrochloric acid solutions, the conductance of the solvent has been subtracted from the measured specific conductances.

Tables I to V give the results of the measurements on the dilute solutions (below 0.005 normal). The average values of the measure-

TABLE VI  
*Equivalent Conductances (25°). Observed and Computed Values at Round Concentrations*

Concn.	Λ, KCl		Λ, NaCl		Λ, HCl	
	Calcd.	Obs.	Calcd.	Obs.	Calcd.	Obs.
0.0001	148.89	148.90	125.54	125.56	424.46	424.53
.0002	148.52	148.56	125.19	125.21	423.82	423.86
.0005	147.77	147.80	124.48	124.50	422.58	422.62
.001	146.95	146.93	123.71	123.72	421.23	421.24
.002	145.81	145.79	122.64	122.67	419.34	419.27
.005	143.65	143.64	120.60	120.58	415.75	415.68
.01	141.36	141.32	118.45	118.43	411.96	411.88
.02	138.39	138.34	115.67	115.65	407.06	407.12
.05	133.33	133.33	110.89	110.88	398.79	398.97
.07					395.42	395.33
.10	128.94	128.90	106.73	106.68	391.78	391.20
$A = 0.2274\Lambda_0 + 59.79$			93.85	88.53		156.70
$B$			94.9	89.5		165.0
$\Lambda_0$			149.82	126.42		426.04

$$\Lambda_0 = \frac{\Lambda + 59.79 \sqrt{C}}{1 - 0.2274 \sqrt{C}} - BC$$

TABLE VII  
*Equivalent Conductances (25°) of Silver and Potassium Nitrates at Round Concentrations*

Concn.	Λ, AgNO <sub>3</sub>	Λ, KNO <sub>3</sub>	Concn.	Λ, AgNO <sub>3</sub>	Λ, KNO <sub>3</sub>
0.0001	132.46	144.02	0.01	124.72	135.78
.0002	132.08	143.53	.02	121.37	132.37
.0005	131.32	142.73	.05	115.20	126.27
.001	130.47	141.80	.07		123.52
.002	129.32	140.54	.10	109.10	120.36
.005	127.16	138.44	$\Lambda_0$	133.32	144.92

ments at the round concentrations between 0.1 and 0.005 normal are given in Tables VI and VII. These two tables also give the con-



ductivity values corresponding to round concentrations between 0.002 and 0.0001 normal, graphically interpolated from the data on the dilute solutions. Graphs corresponding to the data in the tables are shown in Figs. 5 to 8, in which the equivalent conductance is plotted against the square root of the concentration. On these graphs, the dotted lines represent the theoretical slopes ( $\Lambda$ ) calculated from Onsager's conductance equation,<sup>22</sup> which, however, is a limiting expression, strictly only valid at infinitesimal concentrations. *It can be seen from these figures that the Onsager equation yields, in each case, the limiting slope which is approached by the curves representing the actual measurements.*

An empirical extension of Onsager's equation applicable to solutions of strong uni-univalent electrolytes up to about 0.1 normal has been presented in the preceding paper.<sup>23</sup> The equation proposed is

$$\Lambda_0 = \frac{\Lambda + \beta \sqrt{C}}{1 - \alpha \sqrt{C}} - BC \quad (5)$$

which differs from Onsager's theoretical limiting equation by the term  $BC$ .  $\Lambda$  is the equivalent conductance,  $\Lambda_0$  is the equivalent conductance at infinite dilution,  $C$  is the equivalent concentration,  $\alpha$  and  $\beta$ <sup>24</sup> are Onsager's theoretically computed constants depending on the solvent and the temperature, and  $B$  is an empirical constant. As has been shown in the preceding paper,<sup>23</sup> Equation 5 is suitable for obtaining  $\Lambda_0$  values for hydrochloric acid, potassium chloride and sodium chloride, by applying the equation to the conductivity data with  $\Lambda_0$  and  $B$  as the adjustable constants. The limiting values thus obtained agree with the graphical extrapolations, using the measurements on the more dilute solutions in the plots shown in Figs. 5 and 6. The agreement between the observed  $\Lambda$  values for hydrochloric acid, potassium chloride and sodium chloride and those calculated from Equation 5 is indicated in Table VI.

The  $\Lambda_0$  values for the solutions of potassium nitrate and silver nitrate were obtained by linear extrapolations from the plots of

<sup>22</sup> Onsager, *Physik. Z.*, **28**, 277 (1927).

<sup>23</sup> Shedlovsky, *THIS JOURNAL*, **54**, 1405 (1932).

<sup>24</sup> At 25° in water  $\alpha = 0.2274$ ;  $\beta = 59.79$ .

results from the measurements on the most dilute solutions. These plots are shown in Fig. 7. For these substances Equation 5 does not quite fit the observed values. In the case of hydrochloric acid, sodium chloride, and potassium chloride, positive deviations from Onsager's limiting equation, which increase with rising concentration, are observed even below 0.001 normal. However, in solutions of potassium nitrate and silver nitrate the corresponding deviations have small negative values at first and then become positive, but increase

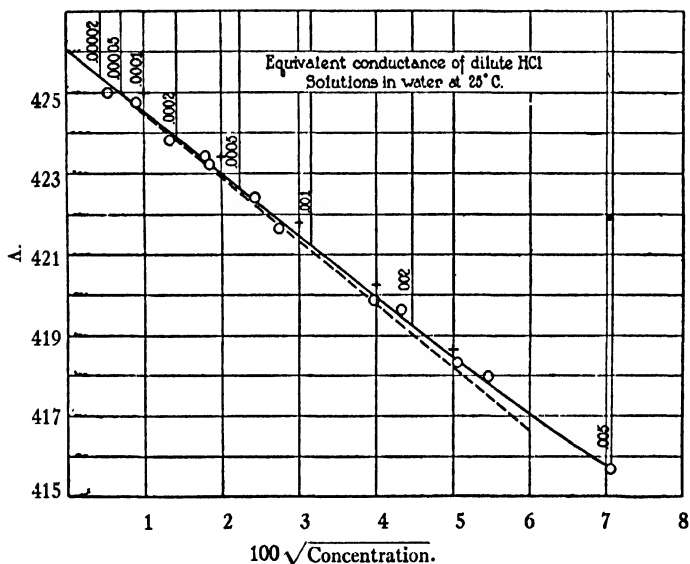


FIG. 6.—O, Shedlovsky; +, Parker

more slowly than is observed for solutions of the chlorides. It therefore appears that a compensating effect is present in these solutions, whose direction is such as might be produced by a slight progressive association of silver nitrate and of potassium nitrate. If this explanation is correct, these salts appear to be somewhat weaker electrolytes than sodium chloride, potassium chloride and hydrochloric acid. However, a more detailed consideration of the conductivity data on silver nitrate and potassium nitrate solutions from this viewpoint will be reserved for a future communication.

In Figs. 5 and 6 some recent measurements at 25° by other workers

are compared with my results. The triangles (Fig. 5) indicate conductance values obtained by Grindley and Davies<sup>25</sup> on potassium chloride solutions, and the crosses, values reported by Jeffery and Vogel<sup>26</sup> for potassium chloride and sodium chloride. The crosses (Fig. 6) indicate values obtained by Parker<sup>27</sup> on hydrochloric acid solutions recomputed to the conductance basis of the present measurements. The discrepancies between the new measurements and those of the other workers are probably due, in part at least, to the use of dipping electrodes, whose disadvantage has been discussed in the

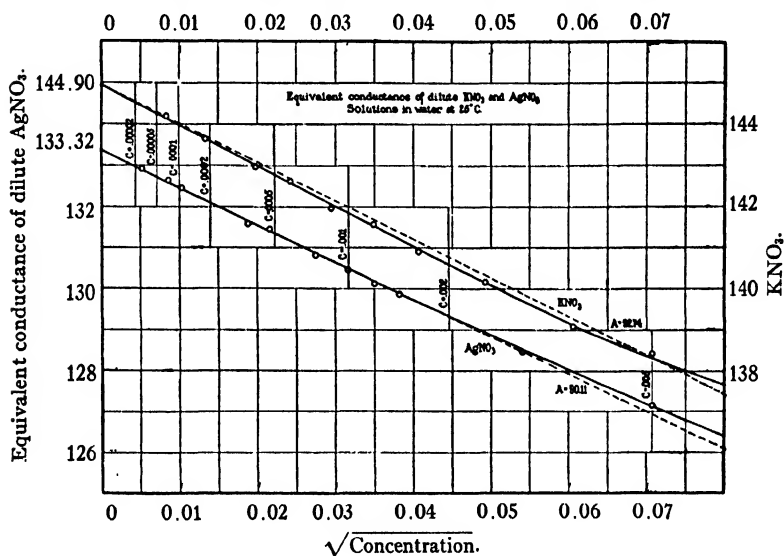


FIG. 7

early part of this paper. In the case of the measurements of Jeffery and Vogel, there may have been also appreciable earth capacity errors, since these authors apparently did not use an "earth-balancing circuit" in their bridge.

*Addendum.*—After this paper had been submitted to THIS JOURNAL, measurements on the conductance of lithium chloride solutions in

<sup>25</sup> Grindley and Davies, *Trans. Faraday Soc.*, **25**, 129 (1929).

<sup>26</sup> Jeffery and Vogel, *J. Chem. Soc.*, 1715 (1931).

<sup>27</sup> Parker, THIS JOURNAL, **45**, 2017 (1923).

water at 25° were completed. The results are summarized in the table below. The equivalent conductance of this salt can be represented accurately up to a concentration of 0.07 *N* by the equation

$$115.00 = \frac{\Lambda + 59.79 \sqrt{C}}{1 - 0.2274 \sqrt{C}} - 89.1 C$$

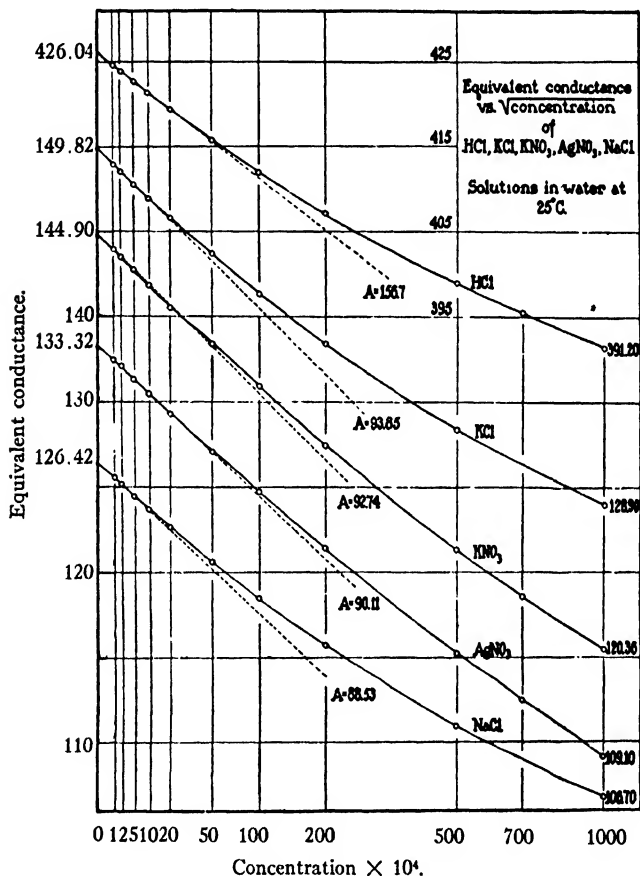


FIG. 8

The salt was prepared as follows. Lithium carbonate was precipitated from a solution of c. p. lithium chloride with redistilled ammonium carbonate. The carbonate was washed several times with hot water and then dissolved in distilled hydrochloric acid. The lithium chlo-

ride thus formed was crystallized, ignited to remove the volatile impurities, and then recrystallized twice from conductivity water acidulated with pure hydrochloric acid. With the aid of the Richards bottling apparatus the final product was fused in platinum in an atmosphere of dry hydrogen chloride and cooled in vacuum. The product was found to be neutral.

*Equivalent Conductance of Lithium Chloride at 25°*

C	$\Lambda$ obs.	$\Lambda$ calcd.	C	$\Lambda$ obs.	$\Lambda$ calcd.
0		115.00	0.032581	102.34	102.30
0.00047210	113.18	113.18	.009836	107.33	107.35
.0023455	111.04	111.05	.018515	104.91	104.92
.0058846	108.91	108.92	.049942	100.10	100.06
.0107786	107.03	107.03	.064021	98.70	98.70
.0199375	104.64	104.61	.10000	95.83	96.19

The author wishes to express his thanks to Dr. D. A. MacInnes for his kind suggestions in connection with this work.

#### SUMMARY

1. The accurate measurement of the conductance of very dilute solutions with a new type of cell is described.

2. It is shown that the use of "dipping electrodes" for accurate conductivity measurements may lead to errors.

3. Measurements of the equivalent conductances of aqueous solutions of sodium chloride, potassium chloride, hydrochloric acid, silver nitrate, and potassium nitrate at 25° are given in the concentration range 0.00003 to 0.1 *N*.

4. These measurements confirm the Onsager conductance equation as a limiting equation.

5. The measurements on the conductances of the chlorides can be expressed, within experimental error, to about 0.1 *N* by an extension of the Onsager equation containing, besides  $\Lambda_0$ , only one empirical constant. The measurements on solutions of the nitrates, however, show deviations from this equation which may be due to partial association.

## THE DETERMINATION OF THE IONIZATION CONSTANT OF ACETIC ACID, AT 25°, FROM CONDUCTANCE MEASUREMENTS

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A number of years ago one of the authors published recalculations of the ionization constants of some weak acids, using the most accurate data then available.<sup>1</sup> In these computations, allowance was made for effects due to interionic attractions. These attractions are functions of the ion concentrations and are made evident by their effects on the ion activities and on the ion mobilities.

In the paper mentioned the law of mass action for the ionization of a weak acid was expressed as follows

$$K = \frac{\alpha^2 \gamma_{H^+} \gamma_{A^-} C}{(1 - \alpha) \gamma_{HA}} \quad (1)$$

in which  $K$  is the ionization constant,  $\alpha$  is the degree of dissociation,  $C$  is the total concentration and  $\gamma_{H^+}$ ,  $\gamma_{A^-}$  and  $\gamma_{HA}$  are the activity coefficients of the hydrogen ion, the anion and the undissociated acid, respectively.<sup>2</sup>

The effect of interionic attractions on the activity coefficients of ions has been dealt with in the well-known papers of Debye and Hückel. The computation of the degree of dissociation,  $\alpha$ , from conductance measurements is influenced by interionic forces in that it is necessary to take account of the effect of such forces on the mobilities of the ions. According to the Arrhenius theory, the degree of dissociation  $\alpha = \Lambda_c/\Lambda_0$  in which  $\Lambda_0$  is the equivalent conductance at the concentration

<sup>1</sup> MacInnes, THIS JOURNAL, 48, 2068 (1926).

<sup>2</sup> Sherrill and Noyes, *ibid.*, 48, 1861 (1926), obtained this equation independently and tested it with data on the ionization of the second hydrogen from dibasic acids. See also Davies, *J. Phys. Chem.*, 29, 977 (1925).

$C$  and  $\Lambda_0$  is the equivalent conductance at infinite dilution. This theory involves the assumption that the ion mobilities do not change with the concentration. To show this let  $U_o^+$ ,  $U_o^-$ ,  $U_o^+$  and  $U_o^-$  be the mobilities of the positive and negative ions at the concentration  $C$  and at infinite dilution. Since

$$\Lambda_c = \alpha F(U_o^+ + U_o^-) \quad (2)$$

and

$$\Lambda_0 = F(U_o^+ + U_o^-) \quad (3)$$

( $\alpha = 1$  in the latter case) then

$$\frac{\Lambda_c}{\Lambda_0} = \frac{\alpha F(U_o^+ + U_o^-)}{F(U_o^+ + U_o^-)} \quad (4)$$

Thus,  $\Lambda_c/\Lambda_0 = \alpha$  only if  $U_o^+ + U_o^- = U_o^+ + U_o^-$ . This can be true only if the mobilities do not change, or by an improbable compensation. Debye and Hückel<sup>3</sup> and more recently Onsager<sup>4</sup> have shown that changes in ion mobilities with concentration are to be expected, on theoretical grounds, even in very dilute ion solutions. Transference data give conclusive evidence that such changes occur at higher concentrations. The original Arrhenius assumption, *i. e.*,  $\alpha = \Lambda_c/\Lambda_0$ , is therefore inaccurate at any concentration.

Equation 2 may be used to compute degrees of dissociation if values of the expression  $F(U_o^+ + U_o^-)$ , which we will call  $\Lambda_c$ , are known. Since  $\Lambda_0$  is the conductance of a mole of acid, the proportion  $\alpha$  of which is in the form of ions,  $\Lambda_c$  is evidently the conductance of a mole of *ions* in a condition in which their mobilities retain the values they have at the concentration  $C$ . Since we have good evidence that strong electrolytes are completely dissociated, at least in dilute solution, values of  $\Lambda_c$  may be readily found from the following considerations. Let NaAc represent the sodium salt of the weak acid (acetic acid for instance) whose degree of dissociation is desired, then

$$\Lambda_{\text{HAc}} = \Lambda_{\text{HCl}}' - \Lambda_{\text{NaCl}}' + \Lambda_{\text{NaAc}}'$$

<sup>3</sup> Debye and Hückel, *Physik. Z.*, **24**, 305 (1923).

<sup>4</sup> Onsager, *ibid.*, **27**, 388 (1926); **28**, 277 (1927).

in which the  $\Lambda_0'$  values are the equivalent conductances of the substances indicated at the ion concentration  $C'$ . For completely dissociated substances this ion concentration is also the stoichiometric concentration, whereas  $\Lambda_{\text{CHAc}}$  is the equivalent conductance of a mole of completely dissociated acetic acid at the ion concentration  $C'$ . Thus for the computation of the degree of dissociation  $\alpha = \Lambda_c/\Lambda_\epsilon$  a value of  $\Lambda_\epsilon$  must be found which corresponds to the ion concentration of the solution of the weak acid whose measured equivalent conductance is  $\Lambda_c$ . This involves a short series of approximations. In the previous paper<sup>1</sup> it was shown that for relatively strong acids, such as *o*-nitrobenzoic acid and *o*-chlorobenzoic acid, with ionization constants  $6.0 \times 10^{-3}$  and  $1.2 \times 10^{-3}$ , respectively, true constants for Equation 1 were obtained over the range of concentrations for which data were available. It was, however, rather disturbing to find that the data for the relatively weak acetic acid indicated a definite trend (from  $1.74 \times 10^{-5}$  to  $1.78 \times 10^{-5}$ ) of the "constant" in the concentration range 0.07 to 0.002 mole per liter. We felt that it was important to find out whether this drift in the ionization constant was due to inaccuracy of the data available or to the assumptions involved in the computation. In addition, an accurate value of the ionization constant of acetic acid is desirable in other connections. It was decided therefore to obtain, with all possible accuracy, data on the conductance of the acid, and of the other substances (sodium chloride, hydrochloric acid and sodium acetate) involved in the calculation of the ionization constant of this acid.

#### EXPERIMENTAL

The conductances were determined with the bridge<sup>5</sup> and cells described in a paper from this Laboratory.<sup>6</sup> The data on sodium chloride and hydrochloric acid used in the computations are also reported in the latter paper. The basis for the conductivity values is that a solution containing 7.47896 g. of potassium chloride in 1000 g. of water (weights in air *vs.* brass) has a specific conductance of 0.012852<sub>4</sub> at 25°.

(a) *Solutions of Acetic Acid*.—C. P. synthetic acetic acid (Niacet Co., U. S. P.) was purified by distilling the material from 2% by weight of potassium perman-

<sup>5</sup> Shedlovsky, *THIS JOURNAL*, **52**, 1793 (1930).

<sup>6</sup> Shedlovsky, *ibid.*, **54**, 1411 (1932).



ganate, using an efficient fractionating column.<sup>7</sup> The product was again distilled from a little chromic anhydride. This was followed by a final fractional distillation. Freezing point measurements were made on the separate fractions. After the first quarter of the material was distilled, later fractions, which were kept for use, gave constant freezing points. The purified acid had a freezing point of  $16.54^\circ$  on a thermometer which had been compared with a carefully calibrated platinum resistance thermometer. The specific conductance of the pure acid at  $25^\circ$  was  $1.4 \times 10^{-8}$  mhos.

We are indebted to Dr. L. G. Longworth, of this Laboratory, for a very careful analysis of this acid by the differential potentiometric method recently described.<sup>8</sup> A solution of approximately 0.2 *N* sodium hydroxide used in the titration was calibrated to an accuracy of better than 0.01% against pure benzoic acid from the Bureau of Standards. This solution, which was nearly carbonate free, was kept in large paraffin-lined flasks which were connected with a carbon dioxide absorbing train. By titration of a stock solution, and of small samples of the acid weighed on a micro balance, the purified material was found to be  $100.04 \pm 0.01\%$  acetic acid. An interesting check on this figure was obtained from the freezing point. Using the cryoscopic constant obtained from De Visser's data<sup>9</sup> and taking Bousfield and Lowry's<sup>7</sup> value of  $16.60 \pm 0.005^\circ$  for the freezing point of the pure acid and the freezing point for our acid given above, the value obtained for the strength of the acid is 100.037%, if it is assumed that the freezing point lowering is due to a trace of acetic anhydride.

To prepare the most dilute solutions it was not possible to weigh out small quantities of the glacial acid with sufficient accuracy. It was, therefore, necessary to prepare stock solutions of about 0.1 *N* concentration, weighed increments of which were successively added to the conductivity water in the cell. A difficulty was encountered in dealing with these acetic acid stock solutions. On making titrations on successive days it was found that the acid was growing weaker at the rate of about 0.01% per day. This was not due to attack on the glass wall of the container as a solution of hydrochloric acid showed no such decrease. The effect was finally found to be due to growth of organisms, probably bacteria, in the acetic acid solution. It was overcome, with the kind help of Dr. Alexis Carrel and assistants, by the use of the following sterile technique.

A solution of approximately the concentration desired was made from the pure acid and conductivity water, and was sealed in a half-filled round-bottomed Pyrex flask. This flask was placed in an autoclave at about 15 pounds' steam pressure for an hour or two on three successive days, to make sure that any spores present were killed. The flask was then opened and the solution was transferred to the flask shown in Fig. 1. This flask had also been sterilized. The transfer took place in a chamber which had been sprayed free of dust. The flask is similar to that

<sup>7</sup> Bousfield and Lowry, *J. Chem. Soc.*, 99, 1432 (1911).

<sup>8</sup> MacInnes and Cowperthwaite, *THIS JOURNAL*, 53, 555 (1931).

<sup>9</sup> De Visser, *Rec. trav. chim.*, 12, 101 (1893).

described by MacInnes and Dole<sup>10</sup> with the addition of a close-grained alundum filter, F. With this arrangement the acid can be forced out by a pressure of gas without fear of contamination by bacteria. Acid, in this flask, kept its concentration to within  $\pm 0.01\%$  for a month.

The data on the conductance of acetic acid are given in Columns 1 and 3 of Table II. Of these data the measurements of solutions having concentrations below  $0.01\ N$  were made in the quartz flask cell<sup>6</sup> with a cell constant of 0.58895. Above that concentration the measurements at round values were made in pipet cells,<sup>6</sup> and the others in a Pyrex flask cell with a cell constant of 8.2183.

(b) *Solutions of Sodium Acetate.*—The solutions of dilute sodium acetate cannot readily be prepared to the desired accuracy in the concentrations by weighing out the dry salt or the trihydrate, since neither of these compounds can be obtained

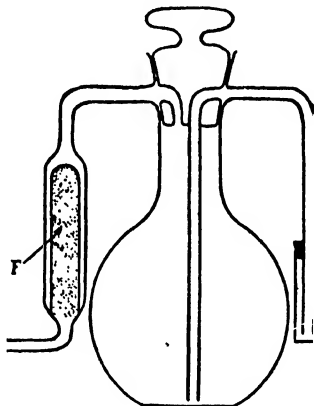


FIG. 1

with sufficient stoichiometric purity. Also it is necessary to control the hydrolysis of the salt. The following procedure was therefore adopted. Sodium bicarbonate, which can be readily obtained free from the potassium salt and other impurities, was heated in an electric furnace at about  $280^\circ$  to yield sodium carbonate. A stock solution of this material (about  $0.1\ N$ ) was prepared and was titrated electrometrically against standard hydrochloric acid, with an accuracy of about  $0.01\%$ . To prepare a solution of sodium acetate from this material, about 1 g. of the glacial acetic acid was weighed out into a quartz ampoule on a micro balance. The necessary amount of the sodium carbonate solution to nearly but not quite neutralize the acid was weighed out into a weighed glass-stoppered flask and the ampoule was dropped into it. The excess of acid was several tenths of a per cent. This flask was then carefully heated to drive off carbon dioxide, which was evolved with

<sup>10</sup> MacInnes and Dole, *THIS JOURNAL*, **51**, 1124 (1929).

practically no spray. After cooling, the flask was again weighed. The concentration of sodium acetate was computed from the known quantity of sodium present. Dilute solutions were prepared by adding small weighed amounts of this stock solution to conductivity water in the cell.

A stoichiometric solution of sodium acetate has the disadvantages that it is considerably hydrolyzed and its conductivity is therefore very sensitive to traces of carbon dioxide, since these would react with the liberated hydroxide. The sodium acetate in a solution containing a slight excess of acetic acid is, however, not hydrolyzed and the ionization of carbonic acid is repressed. The solution is, in fact, buffered against these effects. On the other hand, the conductivity of the solutions thus prepared must be corrected for the conductance due to the very slight ionization of the excess acid. This forms a part of the total solvent correction, which includes the conductivity of pure water in the given solution, and a further correction for conductivity due to a trace of neutral salt if the water had been stored in a Vitreosil container. From  $P_H$  determinations on the solutions the hydrogen-ion concentration may be computed with sufficient accuracy. This figure and the ion product  $(H^+)(OH^-) = K_w$  of water gives the hydroxyl-ion concentration. Since in these measurements the solution was slightly acid, the difference between the hydrogen and hydroxyl concentrations must be balanced by acetate-ion concentration in excess of that arising from the sodium acetate. The magnitude of the correction for the trace of salt was arrived at as follows. The conductivity water from the Vitreosil container also contains a trace of carbonic acid. This was shown in two ways. In the first place, by blowing nitrogen, carefully freed from carbon dioxide, through the water in the conductivity cell the conductance slowly dropped from 3 or  $4 \times 10^{-7}$  to a minimum of about  $1.5$  to  $2 \times 10^{-7}$ . Furthermore, when brom thymol blue, isohydric to  $P_H$  7, was added to a fresh sample of water the color corresponded to about  $P_H$  6.5. However, after the purified nitrogen had been run through the water for about an hour, the color changed to that of  $P_H$  7, and continued passage of gas produced no change. It was thus concluded that the current of gas removed the last traces of carbonic acid and that the minimum conductance measured was due to the pure water plus a minute amount of neutral salt. Since the conductance of pure water at  $25^\circ$  is  $0.55 \times 10^{-7}$ , that due to the trace of salt can be found by subtracting this value from the minimum conductance obtained as described above.<sup>11</sup> The complete solvent correction which was applied to the conductivity measurements of the solutions of sodium acetate was a sum of the neutral salt correction to which were added the conductances of hydrogen, hydroxyl and excess acetate ions com-

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<sup>11</sup> The water used in the measurement of the acetic acid solutions was collected directly into the transparent quartz cell. This water had an initial conductivity  $1$  to  $2 \times 10^{-7}$  lower than the water which had been stored in the Vitreosil container. It has been assumed, therefore, that the salt came from the latter container, and consequently no salt correction was required in the case of the acetic acid measurements.

puted as already described.<sup>12</sup> The  $P_H$  value used in these computations was that of the final solution after the concentration had been built up to about 0.005  $N$  by successive additions of the stock solution. This  $P_H$  was assumed to be constant throughout the measurements since the mixture is a buffer. Also the 0.005  $N$  solution had very nearly the same  $P_H$  as the stock solution, typical values being  $P_H = 6.60$  and  $P_H = 6.72$ , respectively. The  $P_H$  measurements were made electrometrically in the usual Clark apparatus, by Mr. Donald Belcher.

The conductance data on sodium acetate at 25° are given in Table I, which also includes a comparison of the observed values with those obtained from the equation

$$\Lambda_{NaAc} = 90.97 - 80.48 \sqrt{C} + 90C(1 - 0.2274 \sqrt{C}) \quad (5)$$

TABLE I  
*Equivalent Conductances of Sodium Acetate Solutions at 25°*

$C \times 10^4$	$\Lambda$ obs.	$\Lambda$ cal.	$C \times 10^4$	$\Lambda$ obs.	$\Lambda$ cal.
0.99051	90.11	90.17	17.463	87.74	87.76
1.8627	89.92	89.90	20.683	87.52	87.45
5.9058	88.96	89.07	27.973	86.96	86.96
7.3205	88.90	88.86	31.332	86.75	86.74
7.6201	88.76	88.82	39.725	86.28	86.25
14.237	88.03	88.06	41.243	86.16	86.17
15.256	87.95	87.96	43.016	86.07	86.08

This equation is of the same form as those used in expressing the conductance of hydrochloric acid and sodium chloride solutions.<sup>6</sup>

#### DISCUSSION OF RESULTS

As stated in our introduction, the degree of dissociation,  $\alpha$ , is obtained from the ratio  $\Lambda_c/\Lambda_\infty$ , or, possibly more convenient for computation, the ion concentration  $C_i = \alpha C$  may be found from

$$C_i = \frac{1000 \bar{L}}{\cdot} \quad (6)$$

<sup>12</sup> The formula for the total water correction,  $L$ , for dilute solutions of sodium acetate prepared as described above is

$$L = (L_s + 0.350 C_{H^+} + 0.196 C_{OH^-} + 0.041 C_{Ac^-})$$

in which  $L$  is the total correction to the measured specific conductance,  $C_{H^+}$  is the hydrogen-ion concentration  $= 10^{-P_H}$ ,  $C_{OH^-} = 10^{-14}/C_{H^+}$ , and  $C_{Ac^-} = C_{H^+} - C_{OH^-}$ .  $L_s$  is the conductance due to neutral salt. The coefficients of the concentration terms are the limiting conductances of the ions times  $10^{-3}$ .

in which  $\bar{L}$  is the specific conductance of a solution and  $\Lambda_e$  depends upon the ion concentration in the solution. Values of  $\Lambda_e$  (the conductance of completely dissociated acetic acid) as a function of the ion concentration are given by the equation

$$\Lambda_{eHAC} = 390.59 - 148.61 \sqrt{C_i} + 165.5 C_i (1 - 0.2274 \sqrt{C_i}) \quad (7)$$

which was in turn obtained from the equation

$$\Lambda_e = \Lambda_{HCl} - \Lambda_{NaCl} + \Lambda_{NaAc} \quad (8)$$

in which  $\Lambda_{HCl} = 426.04 - 156.70 \sqrt{C} + 165.5 C (1 - 0.2274 \sqrt{C})$ ,  $\Lambda_{NaCl} = 126.42 - 88.53 \sqrt{C} + 89.5 C (1 - 0.2274 \sqrt{C})$  from the accompanying paper by Shedlovsky<sup>6</sup> and  $\Lambda_{NaAc}$  is given by Equation 5 of this communication. This, of course, involves the assumption that Kohlrausch's law of independent ion migration holds at the ion concentrations involved. That no appreciable error arises from this assumption will be shown, from newly obtained data, in a paper to appear shortly from this Laboratory.

To find a value of  $\Lambda_e$  corresponding to an ion concentration  $C_i$ , involved a short series of approximations. A first approximation was made with the limiting value of  $\Lambda_e$ , *i. e.*,  $\Lambda_0$ , giving  $C_i = 1000 \bar{L}/\Lambda_0$ ; from this ion concentration a value of  $\Lambda_e$  was found from Equation 7. A new estimate of  $C_i$  was then made, followed by another of  $\Lambda_e$ , until repetition did not change the result. Usually three approximations were sufficient. From the resulting  $C_i$  values a series of values of

$$K' = \frac{\alpha^2 C}{1 - \alpha} = \frac{C_i^2}{(C - C_i)} \quad (9)$$

were obtained. These are given in Column 5 of Table II. It will be recalled that  $K'$  is the mass action "constant" uncorrected for changes in activity coefficients.

From Equation 1 it may be seen that the thermodynamic ionization constant  $K$  is related to  $K'$  by

$$K = K' \frac{\gamma_i^2}{\gamma_u} \quad (10)$$

in which  $\gamma_i$  is the mean activity coefficient of the ions and  $\gamma_u$  that of the undissociated acid. Thus

$$\log K = \log K' + 2 \log \gamma_i - \log \gamma_u \quad (11)$$

However, from the Debye-Hückel theory in its limiting form

$$-\log \gamma_1 = 0.5065 \sqrt{C_1} \quad (12)$$

and also in the limit  $\gamma_u = 1$ . Thus

$$\log K = \log K' - 1.013 \sqrt{C_1} \quad (13)$$

This limiting equation accounts with surprising accuracy for the measurements at the five or six lowest concentrations, as is shown in

TABLE II  
*Equivalent Conductances and Ionization Constant Values of Acetic Acid at 25°*

Total concn. $C \times 10^3$	Ion concn. $C_1 \times 10^4$	$\Lambda_e$	$\Lambda_c$	$K' \times 10^8$	$K \times 10^8$
0.028014	0.15107	390.02	210.32	1.768 <sup>a</sup>	1.752 <sup>a</sup>
.11135	.36491	389.68	127.71	1.778 <sup>a</sup>	1.753 <sup>a</sup>
.15321	.44049	389.61	112.02	1.777 <sup>b</sup>	1.750 <sup>a</sup>
.21844	.54101	389.49	96.466	1.781 <sup>b</sup>	1.750 <sup>a</sup>
1.02831	1.2727	388.94	48.133	1.797 <sup>a</sup>	1.750 <sup>a</sup>
1.36340	1.4803	388.81	42.215	1.803 <sup>b</sup>	1.752 <sup>a</sup>
2.41400	2.0012	388.52	32.208	1.809 <sup>b</sup>	1.750 <sup>a</sup>
3.44065	2.4092	388.32	27.191	1.814 <sup>b</sup>	1.749 <sup>a</sup>
5.91153	3.1929	387.99	20.956	1.823 <sup>b</sup>	1.748 <sup>a</sup>
9.8421	4.1557	387.61	16.367	1.832 <sup>b</sup>	1.746 <sup>a</sup>
12.829	4.7591	387.41	14.371	1.834	1.743
20.000	5.975	387.05	11.563	1.840	1.738
50.000	9.524	386.19	7.356	1.849	1.721
52.303	9.7542	386.07	7.200	1.854	1.723
100.000	13.496	385.29	5.200	1.846	1.695
119.447	14.763	385.07	4.759	1.847	1.689
200.000	18.992	384.41	3.650	1.821	1.645
230.785	20.371	384.15	3.391	1.814	1.633

Fig. 2, in which the logarithms of  $K'$  are plotted against the square roots of the corresponding ion concentrations. In this plot the solid line  $e$  has the theoretical slope 1.013 of Equation 13. The excellent agreement between the theory and the experimental results for the more dilute solutions is also shown in Column 6 of Table II, in which the values of the thermodynamic constant  $K$  computed from Equation 13 are listed. For the lower range of concentrations these vary but slightly from a mean of  $1.752 \times 10^{-5}$ .<sup>13</sup> Above an acid concentration

<sup>13</sup> In our recent "Letter to the Editor," THIS JOURNAL, 53, 2419 (1931), the slightly lower values of  $K$  resulted from calculations made before our complete data on the conductivity of hydrochloric acid were available.

of about 0.01 *N* the computed values of the ionization constant decrease.

There are a number of reasons why this should be the case. In the first place we have used the limiting form of the Debye-Hückel equation for computing the activity coefficients of the ions. If account is taken of the size of the ions, instead of Equation 13 we have

$$-\log \gamma = 0.5065 \frac{\sqrt{C}}{1 + 0.327 a \sqrt{C}} \quad (14)$$

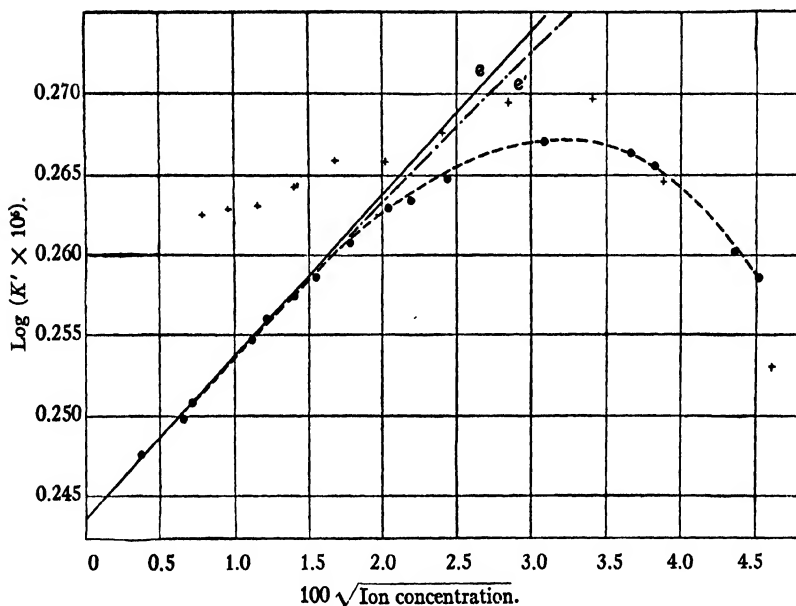


FIG. 2.—●, MacInnes and Shedlovsky; +, Kendall

(where *a* is the distance of closest approach of ions in Ångström units). This equation is valid for the ion concentrations involved in our computations if *a* is greater than 3 Å.<sup>14</sup> In the second place the assumption that the activity coefficient of the undissociated acid  $\gamma_u$  is unity is true only at sufficiently low concentrations. The correction for this deviation is relatively small. It can be evaluated, however, from

<sup>14</sup> Gronwall, La Mer and Sandved, *Physik. Z.*, 29, 358 (1928), give a further extension of the theory which must be used for small ions of higher valences.

freezing point measurements. as has been shown by Lewis and Randall.<sup>15</sup> Although both of these effects are in the right direction, they are not nearly large enough to account for the observed deviations. This is shown in Fig. 2 where the broken line  $e'$  represents the variation of  $K'$  which would be expected if the activity coefficients were modified to allow for the two foregoing effects. In this calculation it has been assumed that  $a$  is of the order of 4 Ångström units, as can be roughly estimated from crystal structure data. It will be observed that the effects of ion size and activity coefficients of the undissociated material are not sufficient to account for the variation of  $K'$  observed.<sup>16</sup>

In computing  $K$  it has been assumed that the accumulation of undissociated acetic acid with increasing concentration has no effect on the solvent. The properties of undissociated acetic acid are, however, quite different from those of water. For instance, the acid has a low dielectric constant, less than 10 per cent. of that of water, and its presence in undissociated form in the aqueous solutions would be expected to influence the *mobilities* as well as the activities of the ions. In our computation it has been necessary, with the data at hand, to assume that the solvent is pure water. This is evidently incorrect at the higher concentrations, and will lead to greater errors the weaker the electrolyte. We expect, in the near future, to study the effect of the change of the medium on the mobilities of the ions.<sup>17</sup>

<sup>15</sup> Lewis and Randall, "Thermodynamics," McGraw-Hill Book Co., 1923, pp. 284, 290.

<sup>16</sup> The change of the activity of the solvent must also be considered at higher concentrations since the ionization in its simplest terms is



Also see Kendall, *THIS JOURNAL*, 39, 2323 (1917).

<sup>17</sup> Davies [*Phil. Mag.*, 4, 244 (1927)] finds that a constant can be obtained from Kendall's conductance data on acetic acid if the conductances are multiplied by the viscosity at each concentration. However, he obtains a higher value than we do for the constant; furthermore, he uses an empirical value of  $A = 0.393$  in the limiting equation  $-\log \gamma = A \sqrt{C}$  instead of the theoretical value  $A = 0.5065$  obtained by Debye and Hückel. He considers that this observation supports Milner's theory (which leads to a lower value of  $A$ ) rather than the Debye-Hückel theory, and also that it sustains Nonhebel's experimental value of  $A = 0.39$  obtained from c. m. f. data [Nonhebel, *Phil. Mag.* 7, ii, 1085 (1926)]. Kendall's data may be readily shown to be of insufficient accuracy to decide the question. In Fig. 2 values of  $K'$ , which have been calculated from Kendall's data, are compared with our results. In these computations his conductances for acetic



Referring to Fig. 2 it is seen that line  $e$ , as has been mentioned, represents the Debye-Hückel theoretical limiting slope, *i. e.*, the constant  $2A = 1.013$  in the expression  $-\log \gamma_1^2 = 2A \sqrt{C_1}$ . Although this line is evidently in close agreement with the data for the lower concentrations, it is nevertheless a limiting equation, and deviations from it must be considered for obtaining the most probable value of the constant  $K$  from the data. Accordingly least square computations were made on the eight and ten lowest points, using the equation

$$\log K' = \log K - 2A \sqrt{C_1} + BC_1$$

The results are given below

	$2A$	$B$	$K \times 10^5$
8 lowest points.....	0.9946	-0.023	1.7533
10 lowest points.....	1.041	-0.047	1.7526

Although the computed values of  $2A$  are, respectively, 2% below and 3% above the theoretical value 1.013, the corresponding variations in the activity coefficients, in the ion concentration range of these measurements, are of the order of a few hundredths of a per cent. Thus the values of  $2A$  are in complete agreement with the theory within the precision of the measurements.

These least square computations give us the limiting value of  $K = 1.753 \times 10^{-5}$ , which is also the result obtained in extrapolating from a plot of the  $K$  values in Table II against  $C_1$ . These constants agree excellently with the value  $1.75 \times 10^{-5}$  obtained in a very different manner (from *e. m. f.* measurements on cells without liquid junction) by Harned and Owen.<sup>18</sup>

acid have been reduced to our basis and our data for the other electrolytes have been used. It will be seen that there is a progressive deviation of these values of  $K'$  from ours as the concentrations decrease. This is what would be expected from Kendall's procedure. His measurements on the more concentrated solutions were made first and the concentrations successively reduced by halves, the dilutions being carried out in air in a glass conductivity cell. The most dilute solution thus had the most handling and had the greatest contamination. Also any adsorption of the acid from the concentrated solutions would likewise tend to contaminate the dilute solutions. The apparent need for a viscosity correction to the data, for the more dilute solutions at least, thus arose from experimental error. As Davies has observed, there is no need for such a correction to the data for the stronger acids.

<sup>18</sup> Harned and Owen, *THIS JOURNAL*, 52, 5079 (1930).

## SUMMARY

Conductance measurements at 25° are reported on aqueous solutions of acetic acid in the concentration range 0.00003 *N* to 0.2 *N*, and on solutions of sodium acetate (in which hydrolysis has been repressed) in the range 0.0001 to 0.004 *N*.

With these data and measurements of hydrochloric acid and sodium chloride, given in an accompanying paper, values of the stoichiometric ionization constants ( $K'$ ) of acetic acid at 25° have been computed. In this computation it has been assumed that the strong electrolytes are completely dissociated and that, for the low ion concentrations involved, the Kohlrausch law of independent ionic mobilities holds. At high dilutions the relation between  $K'$  and the true (thermodynamic) ionization constant  $K$ , *i. e.*,  $K = K'\gamma_{\pm}^2$  (in which  $\gamma_{\pm}$  is the mean ionic activity coefficient) is confirmed if values of  $\gamma_{\pm}$  are calculated from the Debye-Hückel theory. In fact the data corresponding to solutions of acetic acid below 0.01 *N* are in quantitative agreement with the theory. Above that concentration a strong "medium effect," which probably has its greatest influence in ionic mobilities, becomes apparent.

From the measurements at the higher dilutions the value obtained for the thermodynamic ionization constant  $K$  is  $1.753 \times 10^{-5}$ , which is in excellent accord with recent determinations by electromotive force methods.



## SOME SINGLE CRYSTAL SPECTROMETRIC DATA ON UREA

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Increase in knowledge of the atomic positions in organic crystals is dependent upon a better understanding of (1) the scattering powers of the light atoms which make up these solids, and (2) ways in which to correct measured reflections for the imperfections in the crystals producing them. The single crystal data of this paper throw light upon both these questions.

Experiments upon the structures of numerous silicates have shown how to estimate the extinction which measures the orderliness of arrangement throughout such crystals<sup>1</sup>). These methods are not applicable to most organic solids because their crystals are too small and so soft and fragile that the necessary sections can not be cut and ground from them. As the following data illustrate, the requisite information can, however, be gained by combining single crystal with powder spectrometric observations.

Spectrographic, Laue photographic and powder spectrometric data have already been used in studies<sup>2</sup>) of the structure of urea. The present experiments are single crystal spectrometric measurements made in the zone about the *c*-axis with the *K- $\alpha$*  lines of molybdenum, copper, nickel and iron. They are the usual integrated reflections obtained with an apparatus which has already been briefly described<sup>3</sup>). The urea crystal was a cylindrical rod about three millimeters long in the direction of the *c*-axis and less than a millimeter in diameter. Mounted on a fine thread of glass set and oriented in a goniometer

1) W. L. Bragg and J. West, *Z. Krist.* **69**, 118. 1928.

2) H. Mark and K. Weissenberg, *Z. Physik* **16**, 1. 1923; S. B. Hendricks, *J. Am. chem. Soc.* **50**, 2455. 1928; R. W. G. Wyckoff, *Z. Krist.* **75**, 529. 1930.

3) R. W. G. Wyckoff, *The Structure of Crystals*, Second Edition (New York 1931), Chapter VIII.

head of standard design, it was at all times completely bathed in the X-ray beam.

The best and most complete spectra were obtained with copper radiation. Though more molybdenum spectra fall within a measurable

TABLE I  
*Observed Intensities of Single Face Reflections from Urea*

Indices	Integrated reflections (in arbitrary units) for			
	MoK- $\alpha$	CuK- $\alpha$	NiK- $\alpha$	FeK- $\alpha$
110	3256	2523	2633	2324
200	700	617	614	555
120	689	691	645	563
220	433	441	452	416
130	—	80	—	78
230	—	37	—	38
400	—	12	—	14
140	—	4	—	—
330	[200]	[200]	[200]	[200]
240	85	95	96	120
340	—	6	—	—
150	—	34	—	—
250	—	11	—	—
440	—	57	—	—

TABLE II  
*Single Crystal Reflection Data from Urea*

Indices	Intensities (NiK- $\alpha$ radiation)	
	Calculated from powder data	Observed from single crystal reflections
110	8047	2633
200	729 <sup>1)</sup>	614
120	847	645
220	[452]	[452]

range, the large angle reflections are too weak for satisfactory recording. With urea the long wave length spectra of copper, nickel and iron are all more intense than those of molybdenum. They can also

1) The published powder intensity is inaccurate and gives a somewhat greater value than this.

be more accurately measured because the absence of intense general radiation in the neighbourhood of the  $K\alpha$  lines gives a much weaker and an essentially symmetrical background. The integrated intensities that have been studied, arbitrarily referred to (330) as standard, are listed in Table I.

Four prism face reflections of  $NiK\alpha$  radiation are recorded among the published<sup>1)</sup> powder data. A series of single crystal intensities for any desired wave length, freed from the effects of extinction, can be

TABLE III  
*Integrated Reflection Intensities from Urea (CuK $\alpha$  Radiation)*

Indices	Intensities		Corrected
	Observed	Predicted from powder data	
110	2523	8906	9404
200	617	829	751
120	691	937	864
220	441	[506]	506
130	80	—	82
230	37	—	37
400	12	—	12
140	4	—	4
330	200	—	212
240	95	—	98
340	6	—	6
150	34	—	34
250	11	—	11
440	57	—	58

found by multiplying the  $F'^2$ 's derived from these powder results by appropriate values of  $\frac{1 + \cos^2 2\theta}{\sin 2\theta}$ . The difference between such ideal intensities and the measured single crystal data shows the strong extinction present in the latter (Table II).

The experiments on silicates<sup>2)</sup> have indicated how the expression  $\rho = \frac{\rho'}{1 - 2g\rho'}$ , where  $g$  is the extinction coefficient,  $\rho'$  is the uncorrected

1) R. W. G. Wyckoff, Z. Krist. **75**, 529. 1930.

2) W. L. Bragg and J. West, op. cit.

and  $\rho$  is the corrected integrated reflection intensity<sup>1</sup>), can be used to give an approximate correction for secondary extinction. The same sort of correction has been applied to the urea data of this paper by seeking for each set of measurements a value of  $g$  which will give the best agreement between powder and single crystal intensities. The coefficients thus obtained are obviously without absolute significance. Absolute  $g$ 's could be derived by referring all the calculations upon integrated intensities to extended faces; they probably would be of little real meaning, however, because secondary extinction undoubtedly varies with the size of small crystal specimens. Its dependence upon wave length, or rather upon the amount of absorption of different wave lengths, is shown by the fact that, after allowing for differences in reflecting angle, (110) is relatively more intense for molybdenum than for iron radiation. The copper reflections of Table I, thus corrected for extinction, are recorded in Table III.

These intensities have been used for a Fourier analysis of the scattering matter projected upon the  $c$ -face. Urea has no center of symmetry but such a projection has the necessary two-fold axis normal to its plane and the structure factors of the prism faces accordingly have only cosine terms whose signs are known from the previous determinations of structure. The results of this analysis calculated by the usual expression<sup>2</sup>)

$$\rho(x, y) = \frac{1}{a_0^2} \sum_0^\infty \sum_0^\infty F'(h k 0) \cos 2\pi (hx + ky)$$

are recorded in Table IV. A plaster model made from these data is shown in Figure 1. The tall peaks are the superimposed carbon and oxygen atoms, the  $NH_2$  groups on their shoulders are defined by the parameter  $u$  in the nitrogen positions

$$V_d(e)^3: u, \frac{1}{2} - u, t; \frac{1}{2} - u, \bar{u}, \bar{t}; u, u + \frac{1}{2}, t; u + \frac{1}{2}, u, \bar{t}.$$

Part of the flatness at the tops of these groups is due to inaccuracies and incompleteness in the observed intensities but some of it probably

1) C. G. Darwin, *Phil. Mag.* **27**, 315. 675. 1914; **43**, 800. 1922.

2) W. L. Bragg, *Pr. Roy. Soc. (A)* **123**, 537. 1929.

3) R. W. G. Wyckoff, *The Analytical Expression of the Results of the Theory of Space Groups*, Second Edition (Washington 1930), p. 74.

is an expression of a real diffusion of electrons within regions surrounding the nitrogen nuclei. Considering the uncertainty that this flatness introduces into its determination,  $u$  evidently should be taken as  $0.145 \pm \text{ca. } 0.005$ . It is interesting to note that though the scattering matter of individual atoms is distributed throughout a considerable volume, molecules are separated from one another by regions of practically zero electron density.

TABLE IV  
*Electron Densities on the Basal Projection of the Scattering Matter in Urea*

	$x \rightarrow$	0	.05	.10	.15	.20	25	.30	.35	.40	.45	.50
$\downarrow y$ 0	1 63	1 42	0 89	0 84	2 07	3 54	3 59	3 88	8 23	15 75	19 80	
.05	1 42											15 75
.10	0 89		2 62		2 31		2 35		3 48			8 23
.15	0 84											3 88
.20	2 07		2 49		1 96		3 16		2 35			3 59
.25	3 54			4 31								3 54
.30	3 59		7 77	8 56	6 94		1 96		2 31			2 07
.35	3 88	6 24	9 41	10 89	8 56	4 31						0 81
.40	8 23	8 61	9 05	9 41	7 77		2 49		2 62			0 89
.45	15 75	13 16	8 61	6 24								1 42
.50	19 80	15 75	8 23	3 88	3 59	3 54	2 07	0 81	0 89	1 42		1 63

	$x \rightarrow$	.12	.13	.14	.15	.16	$x \rightarrow$	0
$\downarrow y$ .34						10 56	$\downarrow y$ .35	3 88
.35			10 88		10 89		.38	5 88
.355				10 93			.40	8 23
.36			10 86				.41	9 64
.37		10 75					.43	12 74
.38	10 02						.45	15 75
							.47	18.16
							.50	19.80

Once  $u$  has been fixed the spectrometer data give information concerning the scattering powers of the atoms in urea. Only  $NH_2$  groups contribute to  $(hk0)$  reflections having one odd and one even index. The  $F$ 's (for  $NH_2$ ) calculated from the observed  $F$ 's of Table VI are shown as dots in Figure 2. The smooth curve of this figure, the  $NH_2$  curve of the previous paper<sup>1)</sup>, is identical with that

1) R. W. G. Wyckoff, op. cit.



found experimentally for  $NH_4$  in  $NH_4Cl$ ). Except for weak reflections in which the error is great, agreement is satisfactory. The remaining spectra are due to contributions from  $C$ ,  $O$  and  $NH_2$ . By deducting the  $NH_2$  component calculated from Figure 2,  $C + O$  points are obtained which are plotted as dots in Figure 3. Several  $F$  curves have been suggested as giving the true scattering power of carbon in organic compounds. The experimental results for the diamond<sup>2)</sup> are, as might be expected, definitely too great. Existing evidence suggests that the semi-empirical curve used in the pre-

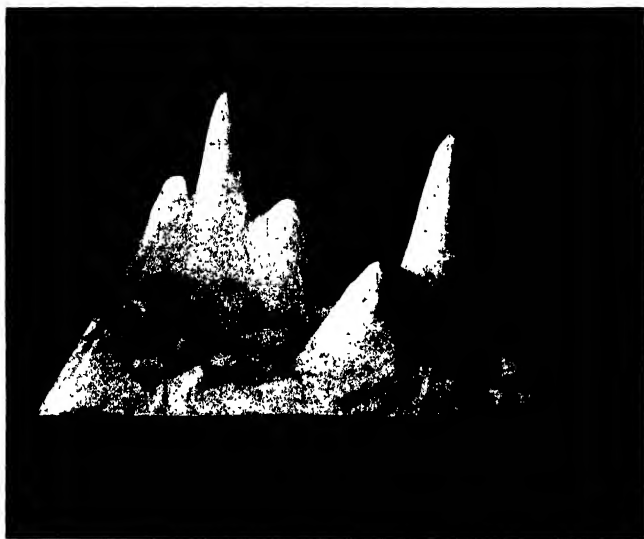


FIG. 1. A plaster model of the scattering matter of urea projected upon the  $c$ -face. Heights are proportional to electron densities.

ceding urea paper, though nearer the truth, still gives scattering powers which are not small enough. The carbon curve of Table V is based<sup>3)</sup> on measurements made with graphite<sup>4)</sup>. As Figure 4 indicates, the oxygen curve obtained by combining the urea  $C + O$  curve with

- 1) R. W. G. Wyckoff and A. H. Armstrong, *Z. Krist.* **72**, 319. 1929.
- 2) A. H. Armstrong, *Physic. Rev.* **34**, 1115. 1929.
- 3) K. Lonsdale, *Pr. Roy. Soc. (A)* **123**, 494. 1929.
- 4) J. D. Bernal, *Pr. Roy. Soc. (A)* **106**, 749. 1924.

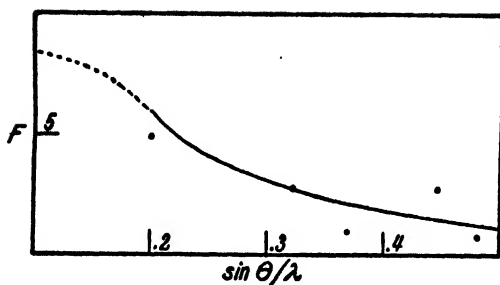


FIG. 2. The scattering powers of  $NH_2$ . The full line is the  $F$  curve of  $NH_4$  from  $NH_4Cl$ ; dots are  $F$ 's of  $NH_2$  provided by the urea data.

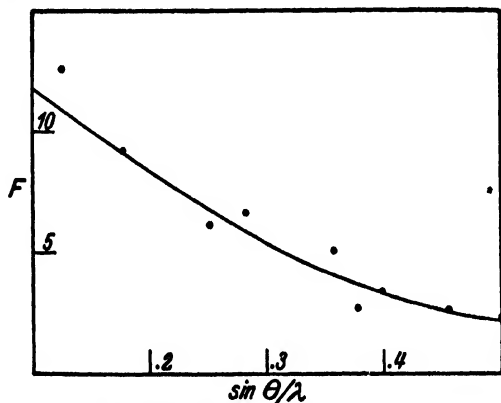


FIG. 3. The full line is the scattering curve of  $C + O$  in urea. Experimental points are shown as dots.

TABLE V  
*Atomic Scattering Powers in Urea*

$\sin \theta/\lambda$	Scattering power of		
	Carbon	Oxygen	$NH_2$
0.12	3.90	7.20	(8.23)
.15	3.34	6.79	(7.68)
.20	2.72	5.78	5.63
.25	2.18	4.67	4.03
.30	1.72	3.63	3.05
.35	1.32	2.98	2.37
.40	1.04	2.36	1.83
.45	0.86	1.74	1.43
.50	0.68	1.50	1.12

these graphite data is in excellent agreement with the oxygen curve found from metallic oxides).<sup>1)</sup> It is therefore to be concluded that

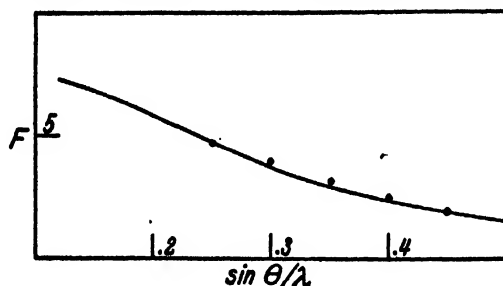


FIG. 4. The full line is the scattering curve of oxygen in urea as calculated from Fig. 3 and the  $F$  curve of graphite. Points on the oxygen  $F$  curve obtained from metal oxides are shown as dots.

TABLE VI  
*Observed and Calculated Reflection  $F$ 's from Urea*

Indices	Observed $F^2$	Calculated		
		$\mu = 0.135$	$\mu = 0.145$	$\mu = 0.155$
110	37.6	36.1	34.1	32.2
200	13.0	15.2	12.0	8.9
120	14.9	16.1	16.5	16.6
220	13.3	13.8	14.5	15.7
130	5.8	4.4	4.2	4.4
230	4.3	6.0	4.1	2.2
400	2.7	0.4	0.5	1.7
140	1.5	1.5	3.1	4.6
330	11.5	12.5	13.7	14.5
240	8.1	7.3	7.9	8.3
340	2.1	0.8	0.9	0.7
150	5.0	3.9	4.7	5.5
250	2.9	3.3	3.6	3.5
440	6.3	7.3	6.7	6.0

the available urea data are in best accord with the atomic scattering powers (for copper radiation) recorded in Table V.

1) R. W. G. Wyckoff and A. H. Armstrong, *Z. Krist.* **72**, 433. 1930; R. W. G. Wyckoff, *Physic. Rev.* **35**, 583. 1930; G. Morton, *ibid.* **38**, 41. 1931.

2) Obtained from the corrected  $Cu$  reflections listed in the last column of Table III.

The extent of the agreement between experimental reflection  $F''$ 's and those calculated from the determined structure is shown in Table VI. It is interesting to see from the  $F''$ 's for  $u = 0.135$  and  $0.155$  that in this instance the Fourier analysis gives a more clear-cut and apparently a somewhat more accurate determination of exact atomic positions than would be furnished by the usual series of structure factor calculations from the same observed intensities.

#### SUMMARY

Single crystal spectrometric measurements with molybdenum, copper, nickel and iron radiations have been made of the ( $h$  $k$ 0) reflections of urea. These intensities after being corrected for extinction using powder spectrometric data have supplied coefficients for a Fourier analysis of the density of scattering matter projected upon the  $c$ -face. The nitrogen parameter  $u$ , as fixed by this analysis, is  $0.145 \pm \text{ca. } 0.005$ . It gives no indication of the separate existence of hydrogen atoms outside of  $NH_2$  groups.

This parameter and the observed reflection intensities have been used to gain information concerning the scattering powers of the atoms in urea. The  $F$ -values of  $NH_2$  found in this way agree with those of  $NH_2$  in  $NH_4Cl$ ; the experimental  $C + O$  curve is practically identical with the sum of the carbon curve from graphite and the oxygen curve from metallic oxides.



## A COMPARISON BETWEEN THE ULTRAVIOLET MICROSCOPY AND THE FEULGEN STAINING OF CERTAIN CELLS

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TER LOUW

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### AUTHORS' ABSTRACT

This paper describes ultraviolet ( $\lambda = 2750 \text{ \AA}$ ) photomicrographs of resting and dividing chicken macrophages and fibroblasts and of erythrocytes and lymphocytes. The structures found in these photographs are compared with the ones brought out in fixed material by Feulgen staining and found to be essentially similar in appearance. A preliminary series of ultraviolet pictures is also shown of a single fibroblast passing through several of its stages of division.

One of the most important advantages of ultraviolet microscopy resides in its ability to show in living material many of the structures ordinarily brought out in stained and fixed preparations. By doing this it can tell conclusively which of the details in the stained figures have reality within the living cell and which are meaningless coagulation artifacts produced by the reactions of fixing and coloring.

Chromosomes are far more opaque to the ultraviolet light used in micrography than are most other constituents of living material. Accordingly it has seemed important to compare ultraviolet photomicrographs for several kinds of cells with preparations stained specifically for chromatin. Some of these results are contained in this paper. In order to be sure that the cells photographed were healthy and not freshly killed or seriously damaged by radiation, series of ultraviolet exposures have been made of a single cell passing normally through the various stages of mitosis.

### *Technique*

The ultraviolet microscope has been used for previous experiments<sup>1</sup> in this laboratory. It consists of a Barnard instrument fitted with

<sup>1</sup> R. W. G. Wyckoff and A. L. Ter Louw, J. Exp. Med., vol. 54, p. 449 (1931).

Zeiss objectives and eye pieces and carrying a modified Leica camera for photography upon motion-picture film. The light source was the 2750 Å line from a condensed cadmium spark, isolated by a simple prism spectroscopic arrangement. The desired field was found with the green line of a mercury arc and sharply focused for 2750 Å by applying an empirical correction. In order to make accurately timed short exposures, a suitable shutter has been interposed between the light source and the condensing lens. Because ultraviolet radiation is destructive to all living cells it is important that material being studied should receive as little as possible of this light. Brief exposures are also essential because under existing experimental conditions a limit to the detail seen in living matter is set not so much by the resolving power of the optical system as by movement within the cells themselves. For both these reasons the final photographs are enlargements from very fine-grained negatives of relatively low initial magnifications.

Four cell types, all from the chicken, were examined—fibroblasts, macrophages, lymphocytes, and erythrocytes. The first two were studied in pure culture, cultivated by the usual methods. The others were found in adult chicken blood. Examples of these cells also were photographed in fresh preparations of embryonic spleen. Most of the photographs of lymphocytes and red cells were obtained from suspensions in Tyrode solution that had been placed between a quartz cover-glass and slide and sealed with vaseline or paraffin. The pure strains and spleen tissue were grown within small containers formed by covering both faces of a drilled glass slide with quartz cover-glasses. The plasma clot in which these cells proliferate must be as thin as possible because of its strong ultraviolet absorption.

The Feulgen reaction<sup>2</sup> was chosen as the means of staining chromatin for comparison with ultraviolet photographs. Samples of each cell type were fixed in alcohol and acetic acid, hydrolized in HCl, and stained with the monaldehyde derivative<sup>3</sup> of sulphonated parafuchsin. Under this treatment formed chromatin takes on an intense violet coloration. Photographs of some of these stained preparations

<sup>2</sup> B. Lee, *Microtometist's Vade-Mecum*, 9th edition (Philadelphia, 1928), p. 306.

<sup>3</sup> E. Wermel, *Zeit. f. Zellforsch. u. mikroskop. Anat.*, Bd. 5, S. 400 (1927).

were made with green light from a mercury arc using a photomicrographic camera equipped with a Zeiss 3 mm.-N.A. 1.4 apochromat objective and an 8 $\times$  Leitz periplan ocular.

Prints from typical photographs with ultraviolet light upon living and with green light upon stained material are reproduced in plates 1 to 3. In most instances these prints were made from duplicate negatives using process film. Additional details concerning individual photographs are to be found in the accompanying descriptions.

Sequences of ultraviolet photographs showing a cell passing through several stages of mitosis were made for both fibroblasts and macrophages. Part of such a series, sufficient to prove that cells are not killed by the amount of ultraviolet light necessary to photograph them, is shown on plate 3.

#### DISCUSSION

In resting fibroblasts the nucleolus alone appears to absorb ultraviolet light. Even so, its opacity is much less than that of the chromatin of dividing cells. The nucleus itself, though it can be seen by reason of the different texture of its protoplasm and the absence within it of the many droplets which fill a large part of the cytoplasm, has practically the same transparency as the rest of the cell. Within the nucleus, however, irregularly outlined specks of absorbing material are often seen. It is important to notice that similar bodies are the only elements which appear on Feulgen staining (fig. 5). The nucleolus does not give the Feulgen reaction but counterstains with the cytoplasmic dye light green (figs. 6 and 7).

Fibroblasts are not readily killed by ultraviolet light. This is clearly shown by the apparently healthy condition of cells which have received for hundreds of seconds the concentrated illumination from the microscope condenser (fig. 3). With too much irradiation, however, they disintegrate by the rupture of their walls and the extrusion of increasingly large masses of protoplasm. A blister of this sort is shown in figure 4.

Macrophages are far more sensitive to ultraviolet radiation. The cell of figure 12 is already ruptured at several points; complete disintegration followed soon after this photograph was made. In resting macrophages, as in fibroblasts, the nucleus and its surrounding cyto-



plasm are about equally absorbing to ultraviolet light. Corresponding to this nuclear transparency is the absence of an intense Feulgen staining (fig. 14) except for a few isolated particles which also appear black in the ultraviolet photographs.

The ultraviolet opacity of the formed chromatin in dividing fibroblasts and macrophages is in striking contrast to these transparent nuclei. The rest of a cell does not seem to become more transparent as absorbing chromatin forms within it. This suggests that a chemical reaction productive of the strongly staining and absorbing chromatin occurs during the early stages of mitosis.

Unlike fibroblasts and macrophages whose nuclei are transparent except during division, the non-dividing erythrocytes and lymphocytes of the chicken have nuclei which at all times contain strongly absorbing bodies. This is true of cells both from early embryos and from adults. Feulgen staining brings out exactly the same structures in these cells (figs. 11 and 15). It will be of interest to determine whether all nucleated non-dividing cells have similar permanent chromatin networks.

The cytoplasm of fresh red cells is moderately absorbing for ultraviolet light. After some exposure to radiation, however, it becomes nearly transparent (fig. 9). This bleaching is scarcely to be explained as due to protoplasmic losses through a ruptured cell wall since the cell outline remains unaltered.

We are indebted to Dr. A. Carrel for the interest he has taken in the carrying out of these experiments.

#### CONCLUSIONS

Ultraviolet ( $\lambda = 2750 \text{ \AA}$ ) photomicrographs have been made of resting and dividing chicken macrophages and fibroblasts and of erythrocytes and lymphocytes. These pictures have been compared with similar fixed preparations stained by the Feulgen reaction. All the gross structures brought out by Feulgen staining have been found in living material by their intense ultraviolet absorption. Although this material is not suitable for comparisons of the finest detail, no significant differences were discovered between the living and the fixed preparations.

The nuclei of the non-dividing erythrocytes and lymphocytes of

the chicken are at all times filled with more or less continuous filaments of chromatin. No absorbing matter can be seen in resting fibroblasts and macrophages but as mitosis begins, intensely absorbing chromosomes gradually make their appearance. The nucleolus is the only other cell organ which absorbs ultraviolet light. Its absorption is, however, so much less intense than that of formed chromatin that there is little chance of confusion. For these cell types, then, ultraviolet microscopy can be considered as the equivalent of a vital staining specific for formed chromatin.

Series of pictures of a single cell passing through its several stages of mitosis show that material photographed by ultraviolet light is capable of continued normal growth and multiplication. It is obvious that this ultraviolet 'motion picture' technique can provide definite and conclusive information concerning many details of cell division.

#### EXPLANATION OF PLATES

##### PLATE 1

1 A fibroblast from embryonic chicken spleen fixed in a thin plasmatic clot and flooded with Tyrode solution. Incubated twenty-four hours and photographed (with ultraviolet light) at room temperature. Previous exposure: eight seconds.  $\times 1200$ .

2 A cell from a subculture of a nineteen-year-old strain of fibroblasts ('Old Strain') photographed (with ultraviolet light) at room temperature. Previous exposure: fifteen seconds.  $\times 1200$ .

3 A fibroblast from embryonic chicken spleen photographed (with ultraviolet light) at room temperature. Previous exposure: 240 seconds. Even after this heavy irradiation the cell showed no sign of breaking up.  $\times 1200$ .

4 A fibroblast from embryonic chicken spleen photographed (with ultraviolet light) at room temperature. Previous exposure: 320 seconds.  $\times 1200$ . After this prolonged irradiation many neighboring cells showed blisters similar to the one in this photograph. The nucleolus, which at first was difficult to see with ordinary light, was easily visible at the end of the experiment.

5 A cell from a subculture of 'Old Strain' fibroblasts fixed and stained with Feulgen reagent and counterstained with light green. The photograph was made on a Wratten 'M' plate with light from a mercury arc filtered through a Wratten no. 77 filter. Little more than the nucleus is shown.  $\times 1200$ .

6 A fibroblast near the one photographed in figure 5. The same technique has been used. In this figure the green-staining nucleoli can be faintly seen.

7 The same cell as the foregoing photographed with mercury light through a Wratten no. 77 filter and a gelatin film stained with eosin and picric acid to bring out more clearly the green-staining nucleoli.

## PLATE 2

8 Adult chicken erythrocytes mounted in Tyrode solution and photographed at room temperature with ultraviolet light. Previous exposure: six seconds. After exposure the irradiation was continued till it totaled thirty-five seconds. Examined then with visible light, the cells appeared normal.  $\times 1200$ .

9 The same cells as those of figure 8 photographed (with ultraviolet light) after ten minutes. The increased transparency of the cytoplasm is evident. After thirty-five seconds, additional exposure to ultraviolet, the cells showed no further change.

10 Embryonic chicken blood cells photographed (with ultraviolet light) by the same technique followed for the two preceding figures. Previous exposure: ten seconds. Structure can be seen in the formed chromatin in the nuclei of both kinds of cells.

11 An adult chicken erythrocyte fixed, stained by the Feulgen reagent, counterstained with light green, and photographed using mercury light filtered through a Wratten no. 77 filter.  $\times 1200$ . The nuclear structure is like that to be seen in the ultraviolet pictures.

12 A macrophage from embryonic chicken spleen photographed (with ultraviolet light) at room temperature. Previous exposure: 150 seconds applied intermittently—ten-second exposures at intervals of five seconds. The cell has already begun to disintegrate; in the first photographs its outline was regular. The nucleus is faintly to be seen in the upper left-hand part of the cell.  $\times 1200$ .

13 Adult chicken leucocytes suspended in Tyrode solution and photographed at room temperature with ultraviolet light. Previous exposure: three seconds.  $\times 1200$ .

14 A macrophage from embryonic chicken spleen fixed, stained by the Feulgen reagent, counterstained with light green, and photographed as usual with mercury light filtered through a Wratten no. 77 filter. Little but the nucleus can be seen.  $\times 1200$ .

15 Embryonic chicken leucocytes from the slide of figure 14 fixed, stained, and photographed as before. The nuclear structure is the same as that appearing in the ultraviolet photograph of figure 13.

## PLATE 3

All of the following figures are ultraviolet photographs of fibroblasts growing in plasma. The preparations were transferred directly from the incubator to a warm stage ( $40^{\circ}\text{C}$ .) of the microscope to induce cell division. The cell of the last figure is from embryonic chicken spleen, the others are from subcultures of the 'Old Strain.' The structures brought out by the Feulgen staining of dividing fibroblasts are seemingly identical with those shown in these figures.

16 to 20 A series of photographs of two fibroblasts taken at intervals of from ten to twenty seconds. Total exposure: eighty-four seconds.  $\times 720$ . Stages in the development of the mitotic figure in the lower cell can be readily followed.

The future course of the division was observed with visible light. One daughter cell disintegrated when the two cells pulled apart, the other became a resting fibroblast of apparently normal appearance. The life history of the upper cell is unknown.

21 The two cells resulting from the division of the lower fibroblast of the preceding figures. The disintegrating cell is at the top; the bottom one later became a normal resting fibroblast.

22 A cell, in metaphase, from the slide used for the preceding photographs.  $\times 1200$ .

23 A fibroblast, in anaphase, from the slide used for the preceding photographs.  $\times 720$ .

24 A dividing fibroblast from a twenty-four-hour culture of embryonic chicken spleen.  $\times 1200$ .





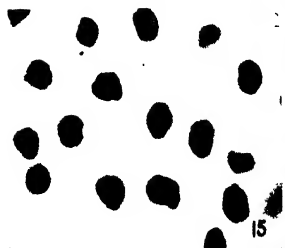
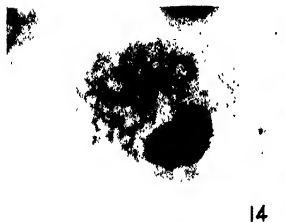
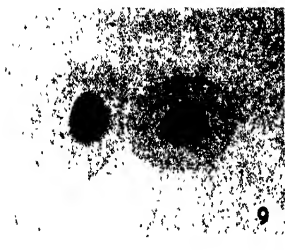
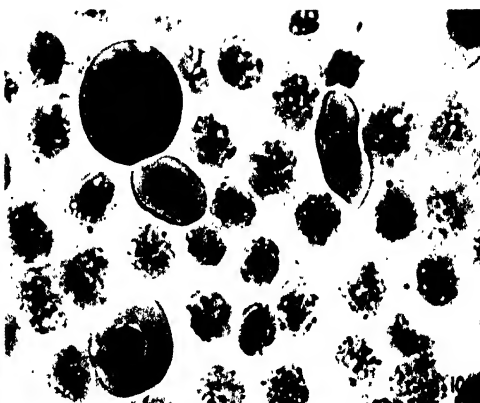
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## THE CRYSTAL STRUCTURE OF THIOUREA

By RALPH W. G. WYCKOFF AND ROBERT B. COREY

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Sound knowledge of the atomic arrangements in crystals of the more important and complex organic compounds can be gained only after a thorough study of several simple aliphatic and aromatic substances. The following determination of the structure of thiourea, as one of these simple compounds, is interesting partly for itself and partly for the understanding it gives of some of the difficulties encountered when quantitative X-ray data are sought from organic crystals.

Two previous investigations<sup>1)</sup> agree in finding four molecules of  $(NH_2)_2CS$  in a unit cell based on the space group  $V_h^{16}$  and having the approximate dimensions

$$a_0 = 5.50 \text{ \AA}, b_0 = 7.68 \text{ \AA}, c_0 = 8.57 \text{ \AA}.$$

In one<sup>2)</sup> of these studies atomic positions have been proposed which are in general agreement with the reflection data from a few simple faces. Though this structure conflicts with the more complete spectrometer data of the present paper it is not far from correct. After rearranging the axes of conventional space group description<sup>3)</sup>,  $X'Y'Z'$ , into the sequence  $Z'Y'X' = XYZ$  to coincide with the crystallographic axes  $a_0b_0c_0$  and after transferring the origin to a center of symmetry, the coordinate positions in this grouping may be written as:

$$\begin{aligned} C \text{ and } S \text{ at } (c) \text{ } uv\frac{1}{2}; \frac{1}{2} - u, v + \frac{1}{2}, \frac{1}{2}; u + \frac{1}{2}, \frac{1}{2} - v, \frac{1}{2}, \\ N \text{ at } (d) \text{ } xyz; x, y, z + \frac{1}{2}; \frac{1}{2} - x, y + \frac{1}{2}, \frac{1}{2} - z; x + \frac{1}{2}, \frac{1}{2} - y, z; \\ x\bar{y}z; x, y, \frac{1}{2} - z; x + \frac{1}{2}, \frac{1}{2} - y, z + \frac{1}{2}; \frac{1}{2} - x, y + \frac{1}{2}, z, \end{aligned}$$

1) L. Demény and I. Nitta, *Bl. Chem. Soc. Japan* 3, 128. 1928; S. B. Hendricks, *J. Am. Chem. Soc.* 50, 2455. 1928.

2) L. Demény and I. Nitta, *op. cit.*

3) R. W. G. Wyckoff, *The Analytical Expression of the Results of the Theory of Space Groups*, 2nd Ed. (Washington, 1930), p. 64, 193.

where for C,  $u = -0.186$ ,  $v = 0.106$ ; for S,  $u = 0.095$ ,  $v = -0.02$ ; for N,  $x = 0.300$ ,  $y = -0.086$ ,  $z = -0.106$ .

The experimental data of the present paper are a few powder spectrometric observations upon very simple faces and single crystal

TABLE I  
*Intensities and Structure Factors for the (hk0) Reflections of Thiourea*

Indices	sin $\theta$	Intensities		Structure factors $F'$		
		Observed	Corrected	Observed	Calculated	Calculated (D. and N.)
110	0.1719	587	830	[36.0]	39.4	31.4
020	.1994	778	1273	48.6	52.4	83.1
120	.2439	593	842	44.2	69.6	67.9
200	.2790	301	354	31.2	-47.4	-32.0
210	.2985	10	10	5.4	-3.9	-3.9
130	.3333	200	222	27.4	34.3	31.5
220	.3458	15	15	7.3	4.2	-1.9
040	.4025	4	4	4.2	-1.3	6.8
230	.4125	15	15	8.2	4.4	0.2
140	.4266	15	15	8.4	3.0	34.1
310	.4337	82	85	20.3	-17.2	6.1
320	.4672	43	44	15.5	-16.2	-7.8
240	.4909	83	86	22.5	22.9	23.2
330	.5188	55	57	19.0	-24.5	-4.7
150	.5222	40	41	16.2	19.3	22.6
400	.5621	57	59	20.5	-17.7	-14.3
410	.5709	< 0.5	0	0	6.9	7.5
250	.5764	7	7	7.1	8.7	9.6
340	.5828	6	6	6.6	5.5	3.5
420	.5964	106	111	29.2	-26.9	-15.7
060	.6032	26	26	14.2	23.8	0.2
160	.6198	5	5	6.3	12.4	5.3
430	.6385	< 0.5	0	0	4.4	—
350	.6565	18	18	12.3	-14.8	-10.0
260	.6663	0	0	0	4.3	—
440	.6917	75	77	25.9	-27.2	-15.5
510	.7098	66	68	24.4	-23.2	-27.3

spectrometric studies of the intensities of all prism face reflections of copper  $K\alpha$ -lines occurring at reflection angles  $2\theta < \text{ca. } 100^\circ$ . The spectrometer used in these measurements and the procedures followed in reducing their results to experimental structure factors have already

been outlined<sup>1)</sup> and illustrated. Single crystals were grown from alcohol and from water solutions. For reflections in the (*hk*0) zone a needle-shaped crystal about 6 mm. long and 1 mm. in diameter was employed. This crystal could be very accurately mounted on an

TABLE II  
*Intensities and Structure Factors for the (0kl) Reflections of Thiourea*

Indices	sin $\theta$	Intensities		Structure factors $F^2$	
		Observed	Corrected	Observed	Calculated
002	0.1800	747	1191	44.4	-65.9
020	.2010	778	1273	[48.6]	52.4
021	.2205	431	549	33.4	-53.8
022	.2702	446	574	39.0	-51.2
023	.3369	55	57	14.1	-21.5
004	.3600	49	51	13.8	13.0
040	.4025	0	0	0	-1.3
024	.4127	151	163	27.2	38.9
041	.4127	2	2	3.0	2.3
042	.4415	55	57	16.9	-27.7
043	.4846	1	1	2.5	2.1
025	.4958	7	7	6.5	10.5
006	.5399	91	95	25.6	-34.3
044	.5406	53	55	19.3	41.8
026	.5768	39	40	17.2	-26.9
060	.6033	10	10	8.9	23.8
045	.6042	0	0	0	2.4
061	.6111	6	6	6.9	17.1
062	.6303	5	5	6.4	-20.2
063	.6618	0	0	0	2.7
027	.6618	0.5	0.5	1.9	7.7
046	.6737	6	6	7.2	-18.3
064	.7039	1	1	3.0	15.7
008	.7199	50	52	21.3	33.6
028	.7483	13	13	10.7	19.5

optical goniometer and gave by far the best data (Table I). For reflections about the *a*- and *b*-axes it was necessary to use platy specimens with large *c*-faces. These crystals were reduced to cylinders

1) R. W. G. Wyckoff, Z. Krist, 81, 102. 1932; The Structure of Crystals, 2nd Ed. (New York, 1931), Chap. VIII.

Electron densities calculated from a series of the usual form

$$\rho(x, y) = \frac{1}{a_1 a_2} \sum_0^{\infty} \sum_0^{\infty} F' \cos 2\pi (hx + ky)$$

are recorded in Table V. Similar densities projected upon the *a*-face are listed in Table VI. The sulfur and  $NH_2$  parameters can be read directly from these tables. From the *c*-face projection  $u_S = 0.12_0$ ,  $v_S = -0.01_0$ ,  $x_N = 0.27_8$ ,  $y_N = -0.13_2$ ; from Table VI,  $v_N = -0.00_8$ ,

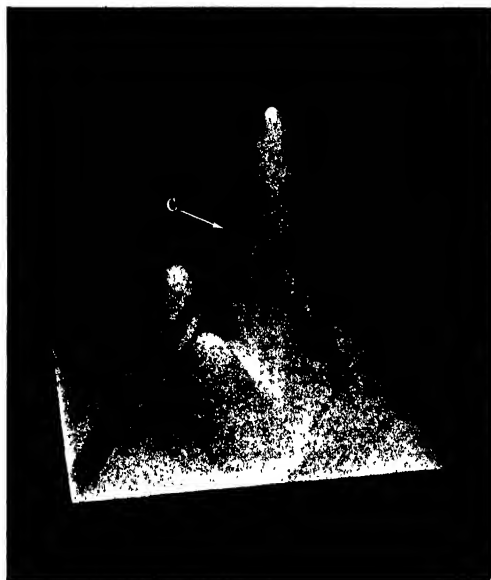


FIG. 1. A photograph of a model showing the electron densities of thiourea projected on an *a*-face (Table VI). The small peaks are  $NH_2$ , the large one is sulfur. The carbon atom at C is merely a hump on the side of the tall sulfur.

$y_N = -0.12_7$ ,  $z_N = -0.12_5$ . The projections of the light carbon atoms lie so close to those of sulfur that they appear only as humps on the larger peaks. This is illustrated by Figure 1 which is a three-dimensional model of the densities of Table VI. If it is assumed that the electron densities of the sulfur atoms are symmetrical about their centers, approximate carbon positions are, however, readily found. They are, from Table V,  $u_C = -0.14$ ,  $v_C = 0.09$ ; from Table VI,  $v_C = ca.$

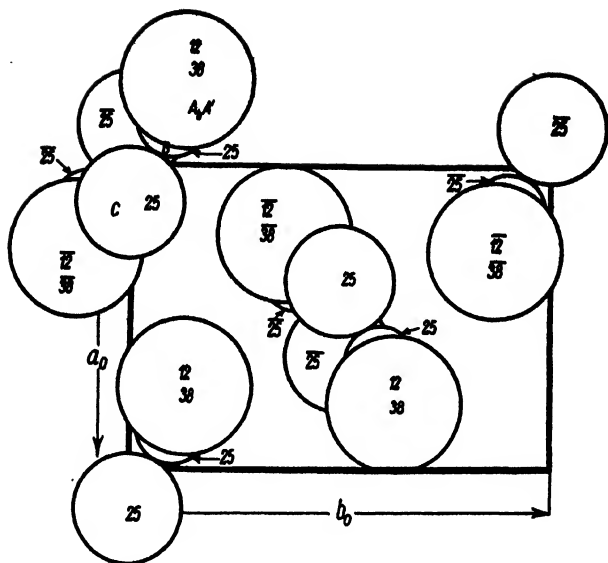


FIG. 2. The structure found for thiourea as projected on its  $c$ -face. The large circles are  $NH_2$ , the intermediate ones sulfur and the small ones carbon atoms. The four atoms  $A$ ,  $A'$ ,  $B$  and  $C$  comprising one molecule lie on nearly a straight line.

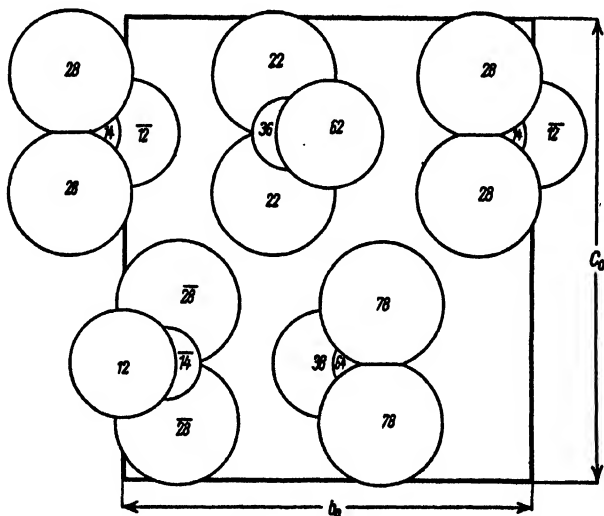


FIG. 3. A projection of the thiourea structure upon its  $a$ -face. The atoms are designated as in the preceding figure and as before the numbers give the parameters normal to the projection plane.



TABLE V  
*Electron Densities Projected upon the ab-Plane as Calculated from a Fourier Analysis of the (hk0) Reflections*

$\rightarrow$ $y$	2	.1	0	.01	.02	.04	.06	.08	.10	.12	.13	.14	.16	.18	.20	.30	.40	.50
$\downarrow x 0$	0.8	-0.3	2.5													1.9	2.3	
.02			3.9						1.9									
.04			7.9															
.06			13.3															
.08			18.6															
.10		2.6	22.1		22.1			11.4	7.2			2.1			1.0	0	+0.8	
.11				23.4														
.12			23.5	23.8	23.5			12.6	8.1	4.9								
.13				23.8														
.14			22.5				16.7	12.5	10.6	5.7								
.16			18.7			17.5	14.8	11.7	8.9									
.18			13.5		14.1	13.6	12.2	10.6	9.2									
.20	1.8	-1.0	8.0		9.0	9.4	9.6	9.7	9.9	10.0		9.6	8.5		4.6	1.3	3.2	
.22			3.4		6.1				11.0									
.24			0.3						12.3									
.26									13.3									
.27											16.5							
.28								10.4	13.8	15.8	16.6	16.0	14.1	10.8	6.9			
.30	1.3	3.2	0			3.4	6.6	10.2	13.2	14.9		14.8			6.4	1.8	-1.0	8.0
.32								9.1									2.6	23.5
.40	0	-0.8	2.5						2.0						0.3	4.2	2.6	22.1

0.11. Hence it must be concluded that the carbon and sulfur atoms in thiourea are in the special positions (c)  $uv\frac{1}{4}$ , etc. of  $V_H^{16}$  with the parameters

$$u_C = -0.14, v_C = 0.10, u_S = 0.12, v_S = -0.007$$

and the nitrogen atoms in the general positions  $xyz$  with

$$x_N = 0.27, y_N = -0.13, z_N = -0.12.$$

TABLE VI

*Electron Densities Projected upon the bc-Plane as Calculated from a Fourier Analysis of the (0kl) Reflections*

$\rightarrow$ s	.25	.20	.16	.14	.13	.12	.10	0
$\downarrow y$ .50							2.0	1.5
.40		1.0					1.4	1.7
.30		0.4					0.6	1.0
.22					2.0			
.20	1.0	1.4			3.2		3.1	1.0
.18					4.6			
.16	2.6				5.7			
.14	3.5		5.1	6.1	6.4	6.4	5.6	
.13				6.2	6.5	6.5		
.12	4.6		5.0	6.1	6.5	6.5	6.0	
.10	6.1	3.9	4.4	5.6		5.8	5.4	1.7
.08	8.2							
.06	10.8							
.04	13.4							
.01	16.0							
0	16.3	7.7					2.0	1.5
-.01	16.1							
-.02	15.6							
-.04	13.4							
-.06	10.2							
-.08	6.8							
-.10	3.9	1.0					1.4	
-.12	2.0							
-.14	1.0							

In the Fourier analysis of urea it was found that the nitrogen peak was decidedly flatter than that of C + O. Similarly the nitrogen maxima in thiourea (Figure 1 and Tables V and VI) are not so sharp as those of sulfur. This fact coupled with the absence of small peaks which could be attributed to hydrogen lends color to the idea that these atoms may

be associated with the nitrogen atoms to form diffuse and unresolvable  $NH_2$  groups.

In comparing the observed structure factors with those predicted by the chosen atomic arrangement, the  $F$ -curves for carbon and  $NH_2$  were the ones found to apply to urea. Since no sulfur scattering curve is available the neighboring one for ionic chlorine<sup>1)</sup> was employed. The calculated  $F$ 's of Tables I-IV refer to a grouping, slightly different from that finally selected, in which  $v_S = -0.00_2$  and  $z_N = -0.12_2$ . Except for the strongest reflections, whose disagreement has already been discussed, the observed and calculated  $F$ 's of Table I are in good quantitative accord. Although quantitative agreement for the ( $h0l$ )

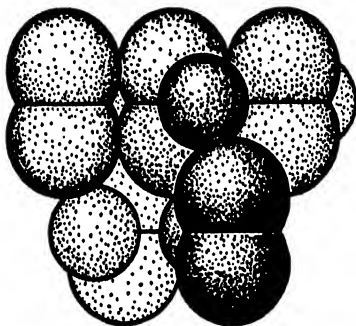


FIG. 4. A packing drawing of Fig. 3 showing how the molecules of thiourea presumably pack together in its crystals.

and ( $0kl$ ) reflections is not to be anticipated because of the inexact crystal settings, the qualitative fit is satisfactory—strong reflections have large calculated  $F$ 's and weak ones small  $F$ 's.

Projections of the structure of thiourea upon its  $c$ - and  $a$ -faces (cf. Tables V and VI) are shown in Figures 2 and 3. There is one important difference between this arrangement and the earlier one. The molecule of Figure 2 has all its atoms,  $A$ ,  $A'$ ,  $B$  and  $C$  for example, in practically the same plane; in the previous structure the molecule was bent so that the angle between  $AB$  and  $BC$  was ca.  $46^\circ$ .

It is of great interest to compare the interatomic distances found for thiourea with those prevailing in other crystals. The  $C-N$

1) R. W. G. Wyckoff, *The Structure of Crystals*, p. 101.

separation within a molecule is almost identical with that in urea  $-1.35 \text{ \AA}$  in  $(\text{NH}_2)_2\text{CS}$ ,  $1.33 \text{ \AA}$  in  $(\text{NH}_2)_2\text{CO}$ . The  $\text{NH}_2\text{-NH}_2$  distance within the urea<sup>1)</sup> molecule ( $2.24 \text{ \AA}$ ) is also nearly the same as in thiourea ( $2.18 \text{ \AA}$ ). For the other intramolecular contact of  $(\text{NH}_2)_2\text{CS}$ ,  $\text{C-S} = 1.64 \text{ \AA}$ , there is no available comparison. Two intermolecular contacts appear to be made in this crystal. Between the  $\text{NH}_2$  groups and  $S$  atoms of adjacent molecules the distance is  $3.45 \text{ \AA}$ . Between the  $\text{NH}_2$  groups, which are nearly if not actually touching, the separation is  $3.85 \text{ \AA}$ . A clear idea of how the molecules of thiourea, given the dimensions indicated by these contacts, pack together to form the crystal can be had from Figure 4. The grouping obviously has nothing in common with that of urea.

## SUMMARY

Spectrometric measurements have been made of the intensities of all the prism face reflections of thiourea having  $2\theta < 100^\circ$ . With the reflection  $F'$ 's arising from these intensities as coefficients, Fourier series have been evaluated giving electron densities projected upon the  $c$ - and upon the  $a$ -faces. The structure thus found is nearly the same as that proposed by Demény and Nitta. Based on  $V_h^u$  and containing four molecules in the unit cell, it is:

$$C \text{ and } S: (c) \text{ } uv\frac{1}{2}; uv\frac{1}{2}; \frac{1}{2} - u, v + \frac{1}{2}, \frac{1}{2}; u + \frac{1}{2}, \frac{1}{2} - v, \frac{1}{2},$$

$$\text{with } u_C = -0.14, v_C = 0.10, u_S = 0.12_0, v_S = -0.00_7,$$

$$N: (d) \text{ } xyz; x, y, z + \frac{1}{2}; \frac{1}{2} - x, y + \frac{1}{2}, \frac{1}{2} - z; x + \frac{1}{2}, \frac{1}{2} - y, z;$$

$$x\bar{y}\bar{z}; x, y, \frac{1}{2} - z; x + \frac{1}{2}, \frac{1}{2} - y, z + \frac{1}{2}; \frac{1}{2} - x, y + \frac{1}{2}, z,$$

$$\text{with } x = 0.27_8, y = -0.13_0, z = -0.12_8.$$

This grouping is pictured and its interatomic distances discussed.

1) R. W. G. Wyckoff, *Z. Krist.*, **75**, 529. 1930.



## THE NATURE OF PNEUMONIA

### THE TWELFTH ANNUAL PASTEUR LECTURE\*

By RUFUS COLE

*(From the Hospital of The Rockefeller Institute for Medical Research)*

The man in whose honor the lectures of this series are given devoted a considerable part of his working life to the elucidation of problems relating to certain infectious diseases afflicting man. It is not unfitting, therefore, that this, the twelfth Pasteur lecture, should relate to one of the most serious diseases which remain to be conquered.

When, after having made his great contributions to chemistry and to knowledge concerning such important biological problems as spontaneous generation and fermentation, and after having applied his discoveries with such extraordinary success to French industries, Pasteur began to investigate certain diseases of man, he was looked upon by the medical profession with distrust, even with hostility, because he was not himself a physician. In 1873, he was elected to the Academy of Medicine by a bare majority of one vote.

However, his own discoveries, and during the succeeding decades the discoveries made by others who were not physicians, in the field of human pathology were so significant that the position of the physician in this domain of science became very seriously threatened. Even today it is with considerable humility, but I must also admit with some satisfaction, that I, a mere clinician, venture to present to you the results of investigations that have been mainly conducted in a medical clinic, and largely by men who are physicians as well as investigators. This satisfaction may be more becomingly expressed because most of the investigations which I shall mention are not my own, but are those of my associates in the hospital of The Rockefeller Institute.

That infectious agents play a rôle in the acute inflammatory diseases of the lungs had long been suspected, but with the advent of the bac-

\* Delivered before the Institute of Medicine of Chicago, November 27, 1931.

teriological era in pathology, this became more firmly established. Today it is assumed that in pneumonia, except under very unusual circumstances, the lesions, the symptoms, and also the events which take place during recovery are directly or indirectly the expression of a reaction between the bacteria and the host. If these reactions, especially those that relate to recovery, are to a considerable degree specific, then classification of the cases on the basis of the nature of the microorganisms concerned in each case would be of great practical importance, and would be essential for interpretation of the disease process.

The introduction of this method of classification, however, has not occurred without difficulties. That bacteria of no single species are concerned in pneumonia became evident very soon after bacteriological methods of investigation were developed. Bacteria of various kinds were found to be associated with those cases in which the lesions in the lung were irregularly distributed, in which the symptomatology was inconstant and varied, and especially in the cases in the very young and very old, or in those occurring in association with other diseases. The situation, however, was different in the cases arising in previously healthy adults, when the lesions in the lung were massive and fairly uniformly distributed, and in which the syndrome of symptoms formed a comparatively uniform picture. Here, in practically all instances, pneumococci were present, and so associated that an etiologic relationship was strongly indicated. There were many confusing features, however, that made the acceptance of such a conclusion difficult. The very frequent presence of these organisms in the respiratory tract of normal individuals, their occurrence in association with other lesions of the respiratory tract, as well as in lesions elsewhere in the body, rendered it difficult to believe that *Pneumococcus* could be considered the specific etiological agent in the group of cases classified together under the designation acute lobar pneumonia.

The discovery, however, that pneumococci are not all alike, in spite of the fact that they appeared to be identical, seemed to offer the possibility that through this knowledge many of the obscure features of the relationship between the microorganisms and the disease might be explained. These differences in pneumococci were

related entirely to the reactions developing in animals into which the bacteria were injected, the reactions which have been very loosely designated as immunological. It was found that the various strains of pneumococci belonged in a number of groups, the microorganisms of each group calling forth identical reactions in the animals into which they were injected. It was soon found that most of the pneumococci associated with cases of the disease having the features usually ascribed to lobar pneumonia belonged in one of three groups, and these organisms were therefore called Types I, II, and III. The other strains of pneumococci called forth reactions which were not identical for any considerable number. With the limited number of these strains available, it was difficult, if not impossible, to classify them, and they were, therefore, as a matter of convenience, called members of Group IV.

On the basis of these facts, and employing the best bacteriological methods available, it has been possible to determine the particular microorganisms present in every case of acute pulmonary disease. So far, it has not been possible to demonstrate the constant relation of any particular feature of the lesions, or any special symptoms, to infection with pneumococci of any single type.

The fact that a very large proportion of the patients with pneumonia of all kinds suffer from symptoms referable to the upper respiratory tract before the signs and symptoms of pulmonary disease become manifest, suggests that even in pneumonia of the lobar type pneumococci are only secondary invaders in a process inaugurated by another agent, possibly a filterable virus. While further experience may show this to be the case, it seems fairly certain that the pulmonary lesions, the more serious symptoms, and the outcome of the disease, at least in the cases associated with pneumococci of Types I and II, are related directly to the presence of these specific microorganisms.

In favor of the primary relationship of these bacteria, moreover, is the fact that pneumococci of these types are very rarely found in the mouths of normal individuals. On the other hand, pneumococci of Group IV are frequently present in these persons, and those of Type III occasionally. However, Dochez and Avery were able to show that pneumococci of the first two types are not infrequently present in the mouths of persons closely in contact with pneumonia patients, and



the type of *Pneumococcus* isolated under these circumstances is the same as that of the pneumococci present in the patient. Stillman has shown that pneumococci of Types I and II can also sometimes be isolated from the dust of the rooms or houses in which the patients are dwelling, and when several cases of pneumonia occur in the same household, or in close association, the type of *Pneumococcus* concerned is usually the same in all of them. All of these facts taken together have seemed to offer good, if not conclusive, evidence that the occurrence of lobar pneumonia is related directly to the presence of pneumococci of these specific types, especially of Types I and II.

Considerable doubt has recently been cast on the validity of this concept, however, since experimental studies have shown that pneumococci, even those of Types I and II, are not so fixed in their specific characters as was formerly thought.

The chemical basis for the type specificity of pneumococci has been clearly demonstrated by Avery, Heidelberger, and Goebel. It was observed that during the growth of pneumococci specific substances are set free and are present in solution in the fluids of infected animals, or in the artificial culture media in which these bacteria are grown. The presence of these soluble specific substances is made evident by the addition to the fluids containing them of the serum of an animal which has received repeated injections of pneumococci of the corresponding type, or, using the customary terminology, of an animal that has been artificially immunized. Under these circumstances a precipitate occurs, which precipitate contains the soluble specific substance. This reaction is very specific. For instance, if an animal is infected with Type I pneumococci, in the serum of this animal, or in the culture medium in which the organisms are grown, a precipitate forms only when Type I serum is added, but when Type II serum is added there is no reaction, and vice versa.

Certain considerations into which I cannot enter here indicate that the material forming the capsule which surrounds virulent pneumococci like a shell or coating is composed in large part of this soluble specific substance. This capsular material from pneumococci of Types I, II, and III has now been obtained in a fair state of chemical purity, and the extraordinary fact has been found that these soluble substances are not, as might be expected, proteins, but that they are

complex carbohydrates—polysaccharides. The polysaccharide of each type of *Pneumococcus* differs chemically from that of each of the other types, and it is to the elaboration of these chemical substances in the capsule that the various types of pneumococci owe their specificity.

In 1916, Stryker found that when pneumococci of Type I or II were grown in homologous specific immune serum, the organisms no longer formed capsules, they lost their virulence, were no longer specifically agglutinable, and now were readily engulfed by leukocytes in normal serum, whereas previously they had been phagocytal only if they were bathed in homologous immune serum. In 1923, Griffith showed that when pneumococci of one or other of the specific types were cultivated in solid medium containing homologous immune serum two forms of colonies developed, and these he described as rough and smooth, now generally called, for convenience, R and S colonies. He showed that the bacteria of the S colonies retain their original virulence and specific properties, while those of the R colonies have undergone the changes described by Stryker. Many studies of this phenomenon have been made by Blake, Reimann, Dawson, and others, and it is now well established that what has happened to the specific pneumococci when grown under these conditions is that they have lost the property of producing the soluble specific substance, and therefore of forming capsules. It is on the ability to form the capsular substance that the virulence of pneumococci depends. The capsule forms a protective sheath or coating. Stripped of the capsule, as in the R forms, the pneumococci fall a ready prey to the phagocytic cells of the body. Possessing capsules, the pneumococci cannot be phagocyted unless they have been treated with homologous immune serum, in which case the surface of the bacterial cell in some way has been altered. Whether this is due to precipitation of the capsular material, similar to that which occurs in the test tube, or whether some other chemical or physical change has occurred at the surface of the bacterial cell, is not known.

Although R pneumococci, free of their capsules, and therefore non-virulent, have lost their specificity as regards type, they still possess a kind of specificity as regards species. R pneumococci, whether derived from Type I, II, or III strains, are all alike as regards their

specific serum reactions, but they still differ from cocci of other species. This has been shown to be due to the fact that the chief antigenic constituent of the noncapsulated cell is protein in nature, and that this protein is antigenically similar in all pneumococci. For if a rabbit be given repeated injections of R pneumococci, or of a solution of the so-called nucleoprotein derived from these bacterial cells, the serum of this rabbit acquires the property of agglutinating all R pneumococci without reference to the specific type of *Pneumococcus* from which they were derived. Also, when this serum is mixed with a solution of the nucleoprotein derived from pneumococci of any type, a specific precipitate occurs.

Now, whereas the type specific S pneumococci, when grown in homologous type specific immune serum, lose their capsules and become non-type specific, and are therefore changed into R pneumococci, it was found by Avery and Dawson that these R pneumococci may regain their specific properties and again become typical S forms if they are grown in anti-R serum, that is, in the serum of an animal that has received repeated injections of the R organisms. The S pneumococci which grow under these circumstances are always of the same type as those from which the R forms were originally derived. A Type I *Pneumococcus* which had been converted into the non-virulent R pneumococcus again becomes a Type I *Pneumococcus* when under these circumstances it reverts to its original S form.

Still more recent studies, however, have shown that pneumococci of the specific types may undergo even more drastic changes than transformation from the S to the R form and vice versa. It has been found by Griffith and by Dawson that if a very few living R pneumococci are injected into mice, together with very large numbers of killed S pneumococci of a type other than that from which the R forms were originally derived, in many instances there may be cultivated from the mice, after death, pneumococci of the same type as the killed S organisms which were injected. There seemed to be two possible errors in this experiment, first, that the supposedly dead S organisms which were injected were in reality not all killed, and that the cocci which grew in the mouse were not modified forms of the R organisms that were originally injected, but were certain of the apparently killed S bacteria which under the conditions of the experi-

ment had started to multiply. The second possible error was that the animals, although apparently healthy, nevertheless harbored living S pneumococci, and that under the conditions of the experiment these dormant bacteria grew and gave rise to infection. Rigorous controls, however, have been made to insure that all of the S organisms are actually killed. The cultures employed may be heated to 80°C. for 15 minutes, or in the case of Type II and Type III pneumococci, to 100°C. for 15 minutes. Moreover, injection of these heated cultures into mice in large amounts never gives rise to infection. If living S organisms were originally present in the mice, they were in a form that previously has not been known, and it was only under the peculiar conditions of the experiment that they were able to grow.

Both of these possible explanations, however, seem very unlikely, and still more improbable in the light of experiments made by Dawson and Sia. In these experiments very small inocula of R pneumococci were added to suitable culture media containing killed S pneumococci of a type other than that from which the R cells were derived. Under these circumstances, also, S forms developed and again they were of the same type as those of the killed organisms. This is especially the case if a little anti-R serum be added to the culture medium. More important still is the observation of Alloway, not yet published. He has found that a similar transformation may occur when, instead of adding killed S bacteria themselves, a heated cell-free extract of the S bacteria is added. The possibility that fragments of the bacterial cells are present in the filtrate cannot, of course, be excluded. But if the S organisms which grow are derived from the extract of S bacteria, they must be filterable forms, and in addition must be much more resistant to heat (60°C. for 40 minutes) than any forms of *Pneumococcus* with which we are acquainted.

The nature of the substance in the extract which stimulates the R forms to synthesize the particular polysaccharides has not been determined. It is not the soluble specific substance itself, since the simple addition of this purified substance, under the conditions of the experiment, is ineffective in bringing about the transformation. The stimulating substance must, however, in some way be specifically related to the soluble specific substance.

In spite of these experiments, which indicate the possibility of the

transformation of pneumococci of one type into those of another type, other evidence is available which strongly suggests that this does not actually occur in nature, at least not with great frequency. Webster has now studied the flora of the nasopharynx in a series of 105 individuals over long periods of time, in some instances for as long as three and a half years. Pneumococci of one type or another were found at some time in 80 per cent of the individuals studied. Pneumococci of Types I and II were obtained from only one and two individuals respectively, and then only on one occasion. Most of the other pneumococci isolated were either of Type III or belonged in the large Group IV. In 13 individuals, however, non-type specific strains, and therefore presumably R forms, were found. But the study showed no indications of transformation of type. Certain individuals carried pneumococci over long periods of time, and in these individuals the strains isolated on successive cultures were, with rare exceptions, identical. For instance, 52 cultures were made from one individual over a period of three and a half years. Pneumococci were always present, and all the strains isolated were identical as regards type. It was also found that while pneumococci of Types I and II show little tendency to spread from host to host, other strains are apparently readily transferred from one individual to another. The results of these observations are, in general, in harmony with those obtained in studies of the epidemiology of certain animal diseases under experimental conditions. In these animal infections, the strains most able to incite severe disease were found to be those least able to spread from host to host. From all these studies the inference may be drawn that pneumococci do not change in their specific character under ordinary conditions, and that in man the incidence and spread of pneumococci of the different types is determined chiefly by differences and variations in host resistance to these strains. According to this hypothesis, pneumococci of Types I and II do not readily spread, because the resistance to infection with pneumococci of these types is very widely distributed. When, however, individuals in whom the resistance is depressed by environmental or other agencies are infected, the resulting disease is most severe.

That man is in general fairly resistant to infection with pneumococci seems likely. For although the distribution of pneumococci of Types

I and II is limited, nevertheless, if in most individuals some mechanism of resistance were not active, the incidence of pneumonia would be even greater than it now is, and its epidemic occurrence would be more frequent. As a matter of fact, man is very resistant to most infectious agents. For instance, it is said that even in the worst European epidemics of cholera only 3 to 7 per cent of the population contracted the disease although all were apparently exposed.

Increased resistance to infection can probably never be related to any single property or set of characteristics. Even in lower animal forms, where the conditions are much more simple, it is not infrequently very difficult to determine whether a single characteristic is inherited, that is, determined by the genes, or whether it is the result of environmental factors, and, if so, what these factors are. When heightened resistance is racial, as in the case of negroes to yellow fever, the chances are that it is genetic, and this is also probable in the case of the resistance of certain species of animals, as the chicken, the pig, etc., to infection with pneumococci. Relatively high resistance of the individual, on the other hand, is more likely to be associated with environmental factors.

As is now well known, individual animals, even of a species that is highly susceptible, may by proper methods be rendered very highly resistant to infection with pneumococci of one type, leaving their susceptibility to infection with pneumococci of other types slightly or not at all changed. For instance, if a rabbit be given repeated parenteral injections of Type I pneumococci of gradually increasing size, the animal finally becomes highly resistant to infection with pneumococci of this particular type. The serum of the immunized animal has also acquired the property of precipitating the carbohydrate from Type I pneumococci. The cells of the immunized animals have acquired a new characteristic, or an old characteristic has been modified, through stimulation by an environmental change. This new characteristic consists in the production of antagonistic, or complementary, or neutralizing substances, which we call antibodies, for a particular and specific chemical compound. In the present instance, the production of this particular antibody is an acquired characteristic of the cells. The ability to form antibodies may, on the other hand, be inherited. This ability is, indeed, a very general property of tissue

cells, and is not restricted to circumstances under which immunity to infection will result. When any one of a large class of chemical substances of large molecular structure is brought into the environment of tissue cells, antibodies are produced. That increased resistance results when antibodies for the pneumococcus polysaccharide are formed is more or less accidental. It happens that this particular substance is on the surface of the bacterial cells, and when the substance unites with its specific antibody the phagocytosis of these particular cells is facilitated. If the polysaccharide were contained within the bodies of the bacterial cells, its antibody might have no functional significance.

As I have already stated, the pneumococcus cell is composed in large part of so-called nucleoprotein. Animals which are given repeated injections of this protein also develop antibodies in their blood, but the resistance of these animals has thereby been in no way increased. Similarly, a third constituent of the pneumococcus cell, also a carbohydrate, has been isolated—the so-called C substance. This is present in pneumococcus cells of all types, possibly also in the cell bodies of other Gram-positive cocci. There is no evidence, however, at present, that antibodies to this substance have any significance as regards resistance.

It would not be expected, therefore, that antibodies directed against the constituents of the pneumococcus cells would play an important rôle in natural resistance. Their presence in the naturally resistant animal would suggest that not only the ability to form antibodies had been inherited, but also the property of constantly forming these particular ones, even in the absence of any specific stimulus. Such a situation is not unknown, however, for the sera of animals of certain species naturally contain antibodies for the proteins of the animals of certain other foreign species. Similarly, the presence of the antibodies for the various blood groups is an evidence of such an inherited property.

The surprise that greeted the publication of reports by Bull, Sia and Robertson, and others, that specific immune bodies are present in the sera of certain animals naturally resistant to pneumococcus infection may therefore not have been entirely warranted. These observers have found that the serum from animals of certain species, such as the

chicken, pig, dog, etc., has a definite action on pneumococci of Types I, II, and III, and that in each instance the action is specific. By the addition of the bacteria of any one of these types to the sera, the antibodies for that particular type may be removed, leaving the others unchanged. Doctor Kelley, in our laboratory, has recently made somewhat similar observations. He has found that in the serum of the pig, type specific antibodies, that can be absorbed by pneumococci of the homologous type, are present. They exist, however, in very small amounts, and they differ in certain properties from the antibodies present in the serum of artificially immunized animals. For instance, they can be absorbed, in part at least, by R organisms. Moreover, they are quite unstable, being destroyed by heating the serum one-half hour at 65°C., and also disappearing after the serum has remained 3 to 4 months in the ice box. For the present, therefore, the matter of the significance of these natural specific antibodies must be left open.

Naturally immune animals may possess other mechanisms than the ability to produce antibodies, however, which confer on them the property of resisting infection with pneumococci, and in one instance, at least, the action of these mechanisms may be specifically directed toward pneumococci of a certain particular type. It is a very striking fact that rabbits are resistant to infection with most strains of Type III *Pneumococcus*, strains which are very virulent for other animals. To infection with pneumococci of other types, however, rabbits are very susceptible. Tillett has shown that when these Type III strains are repeatedly injected into rabbits, no specific antibodies for Type III pneumococci and no precipitins for the Type III soluble substance appear in the blood. On the other hand, agglutinins for R cells and precipitins for the nucleoprotein constituent of the cells do appear. In other words, the reaction is exactly that which occurs when R pneumococci, organisms stripped of their capsules, are injected—no lesions or abnormal symptoms follow, and the organisms are quickly phagocyted. The simplest explanation is that rabbits possess some mechanism for stripping the capsular substance from the cell, or, at any rate, of rendering it ineffective as a protective mechanism.

It is quite certain, however, that animals naturally resistant to infection with pneumococci may possess still other protective mech-



anisms which are entirely unrelated to the type-specificity of the bacteria, or to the soluble specific substance. Animals are occasionally observed which possess greatly increased resistance to infection with pneumococci, but in which no specific activity of the blood, either in agglutinating pneumococci or in protecting animals passively against infection, is apparent. By certain methods, moreover, the appearance of this non-specific type of resistance can be induced by artificial methods. Tillett treated rabbits by giving them, during a period of six weeks, a large number of injections (18) of living R pneumococci. These animals were later found to be highly resistant, not only to infection with Type III pneumococci, the type of *Pneumococcus* from which the R bacteria were derived, but also to infection with pneumococci of Types I and II. At present the exact nature of this kind of resistance is unknown.

By certain investigators, heightened activity of the phagocytic cells of the body has been held to be responsible for increased natural resistance to pneumococcus infection. Some writers have gone so far as to believe that the humoral factors play little, if any, rôle in this phenomenon. Some also believe that they have produced this heightened activity by immunizing animals artificially in the ordinary way. The alteration is held to consist either in an increased functional capacity of the phagocytic cells, or in an increased sensitiveness to chemotactic influences, or in changes in both of these directions. That phagocytosis plays an important, even an essential part, in ridding the body of pneumococci, is certain. That naturally immune animals possess a heightened phagocytic capacity, and that in susceptible animals this capacity may be stimulated by suitable measures, is quite possible. Convincing evidence of this, however, in my opinion has not yet been presented.

In the light of these observations and experiments in animals, what is the nature of the natural resistance to pneumococcus infection which man possesses? It is obviously impossible to determine this by direct experiment. The investigation of the problem is also complicated by the fact that unless the observations are made very early in infancy, the possibility always exists that the characteristics on which the resistance depends have been produced or stimulated by environmental influences, especially by repeated mild and even unrecognized in-

fections with the specific pneumococci. Of recent years much attention has been given to alterations in the reaction of the individual, not only as regards increased resistance to infection but also with reference to what is called an increased sensitiveness to bacteria or their products.

A number of methods have been employed for detecting an increased resistance to pneumococcus infection, or immunity, in man. Tests by a number of observers have been made of the protective action of the serum when injected into mice together with doses of pneumococci that would otherwise prove fatal. All of these observers have shown that while the serum of most persons shows no protective power, the sera of certain individuals may exhibit protective power of greater or lesser degree against pneumococci of one or more types. A more delicate method for detecting differences in the serum is that of Robertson and Sia, by which the pneumococcidal power of serum-leukocyte mixtures is determined. Recently, Sutliff and his associates have employed a similar method, but have used whole blood instead of the serum-leukocyte mixtures. They have found that the blood of relatively few persons is pneumococcidal for pneumococci of Type I, while that of almost all individuals exerts some effect on pneumococci of Type II. The blood of an intermediate number of individuals possesses pneumococcidal power against pneumococci of Type III. These writers also noted certain differences with respect to the age of the persons studied. They found that in the blood of infants 1 to 15 months old, pneumococcidal power is rarely observed; with advancing age its frequency increases, while in the aged it is again seldom present. These observations indicate that a limited number of individuals possess an increased resistance to infection with pneumococci, and that in these cases the heightened resistance is type specific, being increased against pneumococci of a certain type and not demonstrable against those of other types.

Other investigators have observed the reactions following the injection into the skin of small numbers of pneumococci, or solutions of the constituents of the bacterial cells, and have drawn conclusions from the character of these reactions regarding the resistance of the individual to infection, or concerning his so-called state of sensitiveness. It is difficult to draw any conclusions from the earlier studies,

since it now seems that the reactions which occur are not the result of the injection of the bacterial cells as such, but are reactions induced by the injection of the various chemical substances of which the bacterial body is composed. Tillett and Francis have shown that at least two types of reaction may be produced in man by the intracutaneous injection of the constituents of *Pneumococcus*. When the specific polysaccharide is injected, if a reaction occurs it consists of a wheal surrounded by an area of erythema and occurs immediately or within a very short time following the injection, and disappears within one or two hours. On the other hand, following the injection of the nucleoprotein, if a reaction occurs it appears only after 6 to 8 hours, reaches its maximum development in 18 to 24 hours, and may not entirely disappear for 3 or 4 days. This reaction resembles that following the injection of tuberculin, and is probably analogous to the so-called Arthus phenomenon.

The experience so far obtained with the reactions following the injection of these two substances into the skin indicates that the reactions occur when antigen and antibody are simultaneously present. In most instances, when the reactions have been positive, precipitins for the corresponding substances have been present in the serum. Only a limited number of tests with purified polysaccharides have been made in normal individuals who have not previously suffered from pneumonia. Tillett and Francis observed only one doubtful reaction among ten persons studied. Finland and Sutliff report that 14 out of 24 normal individuals showed positive reactions, 4 with Type I polysaccharide and 10 with Type II.

On the other hand, it has been found that positive reactions following the injection of the bacterial nucleoprotein may occur in the large majority of normal people. There is little or no evidence, however, that the state of reactivity to the nucleoprotein gives any indication of the resistance of man to pneumococcus infection. On the other hand, positive reactions following the injection of the soluble specific substance indicate the presence of specific antibodies against the specific polysaccharide, and all animal experiments have shown that this means increased resistance to infection with the homologous microorganism.

All these studies indicate that a considerable number of normal

individuals possess some degree of specific resistance against infection with pneumococci of one or the other specific types. It is possible that the number of individuals who possess this kind of resistance is greater than these observations indicate, for the degree of resistance necessary to prevent infection under natural conditions may be very different from that necessary to prevent infection in the laboratory, and this lesser degree of resistance may not be detectable by the methods I have mentioned.

Many years ago I made some experiments which support this hypothesis. A rabbit was given repeated injections of typhoid bacilli, and then time was allowed to elapse until the agglutinins which formed had all disappeared from the blood. This animal was then injected with a very minute number of typhoid bacilli. Agglutinins again reappeared in this animal, while the same number of bacilli injected into an animal that had not been previously treated had no effect. The fact, therefore, that individuals show no specific blood reactions, does not necessarily indicate that they are not potentially resistant to infection with pneumococci of the various types. The fact that the number of individuals reacting positively to these type specific polysaccharides differs at different age periods suggests that this kind of resistance is an acquired one, and may possibly be the result of repeated mild infections. But this evidence is not necessarily conclusive. It is well known that the reactions on which blood groupings depend are usually not obtainable at birth, but become demonstrable during the first or second year, and yet the specific characteristics on which these reactions depend are undoubtedly inherited. However this may be, it seems that the resistance to infection with *Pneumococcus* must be more widespread than these studies regarding resistance to pneumococci of one or other of the specific types indicate. It is not improbable that the kind of resistance which most individuals possess is of a broad general character, possibly resembling that induced in animals by the injection of R pneumococci.

Recently it has been suggested that the reason why certain individuals develop a localized lobar lesion in the lung rather than a more diffuse general one, such as is seen in the cases of so-called bronchopneumonia, is because these individuals, as a result of previous mild infections with pneumococci, have become sensitized to these bac-

teria, and so, when infected, they react in a peculiar manner. This theory has been elaborated especially by Lauche. He thinks certain features of the onset of pneumonia resemble those of anaphylactic reactions. Moreover, lobar pneumonia does not occur in children under five months because they have not had opportunities to become sensitized. And, finally, he states that lobar pneumonia occurs occasionally in newly born infants whose mothers also suffer from pneumonia, and under conditions, therefore, where a transfer of hypersensitiveness from the mother to the child has probably occurred. Certain other observers, however, maintain that lobar pneumonia not infrequently does occur during the first six months of life. Furthermore, other good pathologists have doubted whether it is possible to say from the published reports of the cases of pneumonia in infants which Lauche cites whether these really were examples of lobar pneumonia or not.

As to what is meant by sensitization to pneumococci is uncertain. If by this is meant the condition which arises following the parenteral injection of foreign chemical substances, as a result of which antibodies appear in the blood and the animal reacts with a local inflammatory reaction when injection of the same substance is subsequently made into the skin, then there are some experimental observations which lend support to this hypothesis. For instance, Opie found that "the injection of 0.2 c.c. of horse serum through the thoracic wall into the lung of an immunized rabbit (rabbit immunized to horse serum) caused localized consolidation with leukocytosis and edema surrounding a central focus of necrosis." Julianelle and Rhoads have also recently shown that in rabbits which have received parenteral injections of egg albumin, or of the so-called nucleoprotein of pneumococci, inflammatory reactions may occur in the parenchyma of the lungs when these rabbits are later injected intratracheally with one or another of the respective substances.

Sharp and Blake have also given rabbits injections of live or dead pneumococci, or of the products of their autolysis, and have later studied the reactions in the skin when tested by the injection of small amounts of the autolysate. All but two of the twenty rabbits treated in this manner showed positive reactions. At various periods these animals exhibiting positive skin reactions were injected intratra-

cheally with a solution of autolyzed Type II pneumococci, and after 24 hours were killed. In a considerable number of these rabbits an exudative inflammatory reaction was present in the lungs. In the rabbits showing no skin reaction, and in normal rabbits previously untreated, the intratracheal injections caused no lesions.

It seems quite certain, therefore, that animals having previously received parenteral injections of antigenic substances react with inflammatory pulmonary lesions when these substances are injected into the lungs. Whether the presence of this kind of reactivity is directly related to the development of the lesions of lobar pneumonia, however, is not so clear. If so, it should not be difficult to produce lobar lesions by injections of living cultures into the lungs of animals prepared in this way. Attempts made by Julianelle and Rhoads, however, to do this have so far been unsuccessful. They have also produced an intrapulmonary reaction by the injection of pneumococcus protein into the lungs of previously prepared rabbits, and on the following day, when the reaction was at its height, they have infected the rabbits. No differences in the course of the infection could be observed, however, in the treated and in the untreated animals.

That localization of the lesion may occur at the site of injection of virulent microorganisms in animals which are naturally, or have been rendered artificially, resistant has long been known. In 1904, Wadsworth showed that if a moderate grade of resistance be produced in rabbits by artificial immunization and if then pneumococci be injected intratracheally, it was possible in a considerable number of instances to produce lung lesions resembling those seen in lobar pneumonia in man. He drew attention to the importance of the relationship between the virulence of the microorganisms and the resistance of the host in determining the nature of the lesions developing in the lungs of infected animals.

Interesting experiments illustrating the importance of this relationship are those of Stillman and Branch. They have shown that when mice are exposed to a spray containing living virulent pneumococci, the bacteria usually disappear from the lungs within a few hours, and rarely give rise to a general infection. If the mice have previously been intoxicated with alcohol, however, the pneumococci persist in the lungs for longer periods, and a fatal septicemia not infrequently

follows. However, if instead of exposing normal mice to such a spray they are first rendered actively immune by the spraying method, then intoxicated by alcohol, and finally again exposed to spraying, a considerable number—about one-fifth of them (45 of 250, or 18 per cent)—die with a septicemia, and in about 25 per cent of these dead mice a lobar type of pulmonary lesion is present. These observations indicate that the localization of the lesion in the lung and its lobar distribution are related in some degree to the state of resistance of the infected animal or man.

It is probable, however, that still other factors are concerned in the production of the finer details of the lesion which is probably seen in its characteristic form only in the human subject. The exact nature of these factors is still uncertain, though Robertson, by the intratracheal injection into dogs of pneumococci suspended in a somewhat viscid gelatin or starch medium, has produced lesions which closely resemble those occurring in patients with lobar pneumonia. The exact way in which the viscid character of the menstruum influences the nature of the lesion is not certain. It has been suggested by Coryllos that the occurrence of lobar pneumonia in man is due to the plugging of a bronchus by infected mucus, and that collapse of the lung is present at the onset of the disease. We have been unable to verify this, however, even in cases seen very early.

Loeschke believes that the marked exudative character of the lesion is related to the state of sensitization of the infected individual. As is well known, exudation is a striking feature of the lesions which develop in the skin following intracutaneous injections of antigen in animals which have previously been injected with the same substance. But, as we have said, these skin lesions are truly inflammatory in character, and exudation of greater or lesser degree is one of the cardinal features of all inflammatory reactions. To claim that because a reaction is highly exudative it is therefore probably a reaction of sensitization seems hardly justifiable. Of interest in this connection are the lesions produced in rabbits by Goodner by the intracutaneous injection of pneumococci. The lesions, and the course of the disease so produced, reproduce in many ways what are considered to be the most striking features of lobar pneumonia in man, the lesions in the rabbit being located in the skin instead of the lung. In this

lesion early and widespread edema is a striking feature. Here, however, there seems to be little likelihood that previous sensitization bears an important relation to the character of the lesion. It may occur in animals which, so far as known, have never previously been in contact with pneumococci.

Only further studies will show the exact rôle which the reactivity of the host plays in the development of the lesions in the lung in lobar pneumonia. That it is of considerable significance seems certain. Whether this altered reactivity is related to inherited characteristics or whether it is brought about by previous infections is not certain. Whether this changed state shall be called hypersensitivity or immunity seems unimportant. The evidence seems to point more and more to the identity, or at least very close similarity, of the reactions at the basis of these two phenomena. These two terms imply harmfulness or usefulness to the host as a whole. Whether the altered state of reactivity shall in any particular case result in benefit or harm to the individual may depend on other circumstances. It is not impossible that the development of a lobar type of lesion may really be favorable. Human pulmonary infections with type specific pneumococci of high virulence might lead to death in an even larger number of cases were it not for the peculiar type of reaction which usually occurs in the lungs. One has only to think of the situation in rabbits, when with certain cultures a single organism placed in the nostril leads to septicemia and death. On the other hand, it would require very subtle reasoning to convince a guinea pig in the midst of anaphylactic shock that it is "all for his good," yet most students now believe that this phenomenon is the manifestation of an antigen-antibody reaction.

It is impossible here to mention all the efforts that have been made to refer the symptoms in pneumonia to chemical substances secreted by the bacteria or arising from their disintegration. Up to the present time no convincing evidence has been brought of the production by pneumococci, during their growth, of a specific toxic substance analogous to that produced by diphtheria bacilli, for instance. During the autolysis of pneumococci, however, it has been found that certain substances appear which on injection into animals give rise to definite pathological effects. Such are the hemotoxic substance, the methemoglobin-producing substance, the substance which induces purpura,



which has been studied by Julianelle and Reimann, and the necrotizing substance investigated by Parker. There is as yet, however, no conclusive evidence that any of them play a rôle in producing the lesions or symptoms of lobar pneumonia. It is not impossible that the injurious substances may never be isolated in quantities sufficient to be studied. Pneumonia is essentially a septicemic disease, though in its milder stages the number of bacteria present in the blood at any time may be few. Yet it is obvious that in their metabolic activities they may alter the fluid medium surrounding the cells to such a degree that cellular injury results, without the actual injurious substance being detectable, or, indeed, possibly without specific chemical substances being formed. One needs only to recall the effects of very slight modifications of the medium in which tissue cells grow under artificial conditions, and the accuracy with which the equilibrium of the internal environment is maintained in the normal body, to realize the difficulties which may be inherent in detecting the exact nature of the injurious action of pneumococci growing within the tissues. When we add to this difficulty the possibility that the reaction may depend, to some extent at least, on the specific reactions between the bacteria and the host, depending upon previous interactions, it is obvious that before the solution of this problem is obtained a very long and tortuous path may have to be followed.

It may seem strange that in discussing the nature of pneumonia, I shall give little attention to the symptoms exhibited by the patient. It is not because I do not recognize the importance of the careful observation and description of these features, nor because I do not recognize that through such study information may be obtained which may be of great service in revealing the true nature of the disease. It can hardly be gainsaid, however, that up to the present time, although the accurate description of the symptoms has been of great importance for the physician in enabling him successfully to meet the physiological disturbances which arise, it has not been very fruitful in giving an insight into the underlying phenomena.

Considerable information is now available regarding the nature of recovery from pneumonia. While it is possible, of course, that a symbiotic adjustment between virulent pneumococci and man might occur so that the pneumococci might live in the human body without

producing symptoms, the evidence now available indicates that recovery from pneumonia is accompanied by death of the virulent microorganisms. It is true that virulent pneumococci may persist in focal lesions, as in empyema cavities, for considerable periods of time. But in such cases the body as a whole is apparently highly resistant, and the organisms in these lesions are, in a way, living in regions walled off from the body, and are no longer exposed to the action of the various mechanisms which provide for their removal. An interesting exception to this statement is the phenomenon which has occasionally been observed in horses which have been used in the preparation of immune serum, and which have received repeated injections of living culture. These horses sometimes suffer from chronic endocarditis, and from the lesions in the heart pneumococci may at times be isolated. It is a most interesting and striking fact, however, that these organisms in the endocarditic lesions are R organisms, they are not type specific, they are not virulent, and they do not possess capsules. Exposed as they are to the action of immune serum, it is probable that under these conditions the same factors are responsible for the loss of type specificity as those which bring about this loss when pneumococci are grown in specific immune serum outside the body.

From what has previously been said concerning resistance and immunity in pneumonia, it is apparent that the body possesses a number of mechanisms that might possibly be employed for getting rid of pneumococci that have invaded the tissues, and have given rise to lesions, and it is not impossible that on occasions various of these mechanisms, and possibly others that we know nothing about, are effective. However, following the discovery of the type specificity of pneumococci and the demonstration of soluble specific substances, it was found that during recovery the body elaborates specific antibodies against these soluble substances. The presence of these antibodies during the crisis or during recovery from pneumonia may be demonstrated by several methods. They may be detected by the agglutinating action of the serum on the homologous bacteria or by its precipitating effect when mixed with the soluble specific substance. The presence of antibodies may also be demonstrated by means of the specific reaction in the skin when the soluble specific substance is

injected. Their presence may also be demonstrated by the ability of the serum to confer passive protection against homologous organisms when injected into mice, and also by the action of serum-leukocyte mixtures on homologous pneumococci in vitro.

A considerable number of patients have now been studied during recovery from pneumonia by one or other of these methods. A definite relationship between the disappearance of the organisms from the circulation and the appearance of antibodies directed toward the soluble specific substance has not been found in all cases, but in most instances the parallelism has been so striking as to justify the conclusion that usually these two phenomena are intimately related. That, in the absence of complicating factors, the administration of substances antagonistic to the soluble specific substance is alone sufficient to bring about recovery from virulent pneumococcus infections, is shown by the action of immune serum in infected animals. Finally, the evidence is now fairly convincing, at least to a considerable number of observers, that even in man, recovery in pneumonia, due to Type I pneumococci, at least, may be induced or hastened by the introduction of these substances.

The possibility that the armor of the specific pneumococci may be attacked in other ways than by the action of specific antibodies in the serum has recently been shown by Avery and Dubos. Since the pneumococci owe their virulence to the possession of capsules composed of specific polysaccharides, it seemed that it might be possible to find an enzyme, or enzymes, that would attack these substances and cause their chemical disintegration. After prolonged search, a bacterium has at last been found that has the property of breaking down the soluble specific substance of Type III *Pneumococcus*. From cultures of this bacterium, the specific enzyme responsible for this action has now been obtained in solution, free from the bacterial cells. If a small amount of this enzyme solution is added to a culture of virulent Type III pneumococci in the test tube, these organisms grow, but they have no capsules; the capsular material is destroyed as rapidly as produced. If these pneumococci are removed from this medium and inoculated into an enzyme-free medium, capsules are again formed. It has also been found that the action of this enzyme takes place not only in vitro but also in the animal body. When the

enzyme solution is injected into mice infected with these virulent Type III pneumococci, the organisms disappear and the mice recover. Studies of peritoneal exudates of infected mice, into which the enzyme has been injected, show that its action under these circumstances is exactly the same as it is in the test tube. The capsules of the bacteria are destroyed as fast as formed, and the bacteria freed of their capsules can now be readily phagocyted. Similarly, if rabbits are infected by injection into the skin, so that a chronic infection lasting several days is produced, accompanied by growth of pneumococci in the blood, and if then proper amounts of the specific enzyme solution be injected, the rabbits may be cured of the infection. Whether in pneumonia in man a similar mechanism is ever operative during recovery is not known. As previously mentioned, however, in the recovery of rabbits from Type III infections, a similar process seems to be the usual one.

I have now discussed some of the phenomena which apparently are of importance in the reaction between the human body and the bacterial cells during the course of pneumonia. At present our knowledge of the underlying phenomena is almost entirely confined to those cases in which type specific pneumococci are concerned, and which we call lobar pneumonia. Less is known concerning the other varieties of pneumonia. But it seems certain that as more knowledge concerning the more typical cases is obtained, clues will be found that will be of importance in interpreting the nature of these other conditions. In these atypical cases, however, not only specific factors, but also more general properties of the body, even such factors as the nutritional state, may be of considerable significance. For this reason, the interpretation of the nature of these conditions may be more difficult than in the case of lobar pneumonia. Certainly, to modify the course of the disease by specific measures may be more difficult.

Man lives in a fairly uniform environment, the external one somewhat more variable, the inner one almost constant. His adaptation to his environment is so perfect that any change is potentially harmful, and whenever the variation exceeds the adaptive capacities of the organism, disease results. The animal, however, is well endowed by inheritance with what Doctor Meltzer called "factors of safety" to meet quantitative modifications in the environment. Under or-

dinary circumstances, these quantitative deviations are slight in comparison with the wide variations that are possible.

When bacteria gain access to and grow in the body, changes in the internal environment immediately occur. Certain of these changes, for instance alterations in the acidity of the fluids, are of a quantitative character, and the factors of safety are sufficient to meet these emergencies. Other changes may depend on the presence of products arising during the metabolism of the bacterial cells, and here again the factors of safety may be adequate for their decomposition or removal, for to the ordinary products of bacterial metabolism the body may not be unaccustomed. Still other changes, however, are associated with the presence of substances with which the individual or his immediate ancestors may previously have had no experience. If these substances are insoluble, as, for instance, the entire bacterial bodies, or particles of large size, possibly even very minute ones, the phagocytic cells may remove them, provided the physical character of the surface of these particles is suitable.

In addition, however, other substances which are soluble, and are also quite new to the organism, enter the environment. Such are the organic chemical compounds which constitute the bodies of the bacteria. Now, if these substances are not immediately decomposed, they stimulate a very peculiar phenomenon—complementary, antagonistic, neutralizing bodies appear in the serum. This phenomenon is of great biological significance, and probably not merely in its relation to infectious disease.

As you know, the nature of these antibodies is still quite unknown. It is not known whether they are formed and secreted by the cells, whether they are produced in the fluids of the body through the action of ferments originating in the cells, or whether they may arise in serum quite independently of any cellular activity. The best evidence at present available, however, indicates that the tissue cells take part in their production.

In their action they are specific, that is they are reactive only with the substances that stimulate their production. Recent studies, however, show that this statement must be qualified, for it has been found that substances from various and diverse sources may exhibit identical reactions. For instance, the soluble substance obtained

from Type II Pneumococcus, that from Type b Friedländer's bacillus, and a polysaccharide obtained from gum arabic, are all precipitated in the sera of animals repeatedly injected with either Type II pneumococci or Type b Friedländer's bacilli. These substances all resemble one another chemically, but they are not chemically identical. It is now known that the specificity of the antibody is not necessarily directed toward the whole molecule of the substance with which it reacts, but that its specificity may be determined by as minute a peculiarity in the molecule as the spatial position of simple groups on a single carbon atom. This has been shown by Avery and Goebel for certain glucose protein compounds, the remainder of the molecule being of somewhat minor importance so far as this particular reaction is concerned. It is no longer necessary, therefore, to compare the relation of the antibody with its antigen to the relation of a very complicated key with its lock or of a hand with a glove. .

The most remarkable fact about the phenomenon, however, is not its specificity—all chemical reactions show some degree of specificity—but it is the fact that the production of the reacting substance, so far as known, occurs only in the presence of living matter. The formation of antibodies is a general biological phenomenon. It occurs under all sorts and varieties of conditions. Like other biological phenomena, it undoubtedly had a purposeful origin, but it is a physico-chemical reaction which in any particular instance occurs independently of whether its effect on the whole organism is beneficial or not. Probably the acquirement of knowledge concerning antibodies has been delayed by the fact that the chief interest has been centered in the phenomena resulting from their reactions with antigens, and on the teleological significance of these reactions, or on their usefulness. Immunity of the host against bacteria, or even against disease, has been spoken of as if the animal reacted in some way with the entire bacterial cell. There is little evidence for this conception.

Bacterial cells, as well as tissue cells, may be stimulated to produce specific reacting substances, and the stimulating factors may also be specific, as is probably the case in the transformation of pneumococci from those of one type into those of another. The differences in these bacteria depend upon the formation by each of them of a different chemical substance. It might be intellectually satisfying to know

that the changes which the bacteria undergo are useful. The loss of the property of forming the specific substance, however, while it might be useful to the bacteria growing in immune serum in the test tube, is certainly not useful to the bacteria growing in the immune animal. Neither bacteria nor tissue cells are able to decide, under any particular circumstances, whether a given reaction will be useful or harmful.

I have touched on these somewhat fundamental matters for two reasons. First, because their consideration brings out in a rather fascinating manner certain analogies which exist between the problems on which Pasteur threw so much light a half century ago, and the problems concerning the phenomenon of antigen-antibody reaction, which are of so much concern today. Pasteur's first interest was in the specific differences between two substances having identical chemical composition, but reacting differently. He showed that the differences depended upon the spatial arrangement of the molecules, one being a mirror image of the other. It is an interesting fact, though one on which he did not lay great stress, that the two substances, dextrotartaric acid and lævotartaric acid, unite to form a compound, the solubility of which is less than that of either of them. In his studies of fermentation, also, Pasteur very soon came in contact with the question of specificity. As he says, "If I place one of these salts of racemic acid . . . in the ordinary conditions of fermentation, the dextrotartaric acid alone ferments. Why?" We now know that the specificity of ferment action may be as great as that exhibited by antibodies. Pasteur was concerned with the constant relationship of fermentation to living things. It remained for Buchner, however, to show that the ferments may be obtained in solution entirely free from the cells giving rise to them. Whether these analogies between the problems studied by Pasteur and those urgently awaiting solution today, or whether the solutions arrived at by Pasteur have any bearing on the present day problems, I leave to you.

The second reason why I have discussed these rather fundamental questions is because of their bearing on the disease we are considering, and their bearing upon the experimental studies I have mentioned. We are very far from knowing the nature of all the events which take place when pneumococci invade the body. But certain of these

events we do now know, either by direct observation or through experiment. To one of these events I have given special attention tonight, because it seems possibly to have a very fundamental significance, not only as regards recovery, but also in relation to the whole course of the disease. This is the antigen-antibody reaction. There may be other phenomena equally or even more significant in pneumonia, but at present we know little concerning their nature.

Three constituents of the pneumococcus cell have now been isolated, one of them in a comparatively pure chemical state. Each one of these substances, when in solution and brought into contact with the cells of the body, stimulates the production of an antagonistic, or reactive, or complementary substance, which unites with it, and outside the body at least, a precipitate is formed. The capsular polysaccharide being on the periphery, and quite soluble, is continuously given off into the surrounding medium. Whether attached to the cell or free in the body fluids, it readily combines with its antibody and thereby its physical properties are altered. As a result, the bacteria become readily engulfed by leukocytes. In this instance, therefore, the result is favorable for the entire organism. During the course of pneumonia, antibody is being produced in constantly increasing amounts, but at the same time the bacteria are producing the capsular material. If there is much of the capsular material in solution in the body fluids, this combines with the antibody and there is less antibody available for the substance attached to the cells.

The outcome of the disease depends, to some extent at least, on whether antibodies are formed in sufficiently large amounts to neutralize the capsular substance in solution, and also to unite with the capsular substance of the bacteria, and thus facilitate their phagocytosis before the effects of the growth of the bacteria are so severe as to render life for the individual impossible. It has been found possible to produce these antibodies in large amounts artificially in animals, and by supplying them to the sick individual to help overcome the infection.

It would be absurd to maintain that the outcome in pneumonia is determined solely by the relative rates of production of the soluble specific substance and its antibody. Other antibodies are undoubtedly being formed. Of some of them we may be quite unaware. Some



of them may be useful. I have also mentioned another kind of reaction which may be taking place, namely the decomposition of the capsular substance through the action of an enzyme. I have described how it is possible to supply this ferment artificially to infected mice and rabbits, and so to bring about or hasten their recovery.

Whether the reactions between antigen and antibody are of significance in determining the localization of the lesion and the symptoms produced is not certain, though there is some evidence that this is the case.

Finally, although I have been seeking a description of certain isolated phenomena occurring during the course of pneumonia, I have not been unmindful of the dictum of Professor Haldane, that in studying biological phenomena "we must keep the whole organism in view"—that the living organism is a whole, not merely a collection of its parts, and that whatever happens in one part of the body affects every other part. Moreover, experience abundantly teaches that conclusions regarding phenomena that occur in the test tube cannot be applied to the living body without reservations, and that what happens in one animal under certain circumstances does not necessarily happen in another under similar conditions. The conditions can never be identical. Biological phenomena can never be fully understood unless in addition to the study of isolated phenomena the "physis" also be studied, and by "physis" the Greeks meant "the organism—the organism as a whole." It is this element that medicine must not disregard, but experience teaches that we can best understand the whole by the study of the parts, not as isolated events, but in their relation to the entire organism.

## ÜBER KATALASEWIRKUNG VON EISENVERBINDUNGEN IN KULTURMEDIEN

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Die Lebensdauer von Pneumococcen ist mit den Oxydationsprozessen der Zellen eng verknüpft. Pneumococcen sterben in gewöhnlicher Bouillon rasch aus; es genügt ein kleiner Zusatz von Blut zu dem Kulturmedium, um sie lange Zeit am Leben zu erhalten. In flachen Schichten an der Luft gezüchtet, bilden sich Peroxyde (Benzidin +  $H_2O_2$ -Reaktion), welche die Lebensfähigkeit der Zelle herabmindern oder sie ganz töten, unter solchen Umständen genügt ein Zusatz von Katalase, um sie am Leben zu erhalten.<sup>1</sup>

In der vorhergehenden Arbeit wurde demonstriert, daß gewisse Eisenoxyde relativ starke Katalaseaktivität besitzen. Es war von Interesse, Eisenoxyde auf ihre Fähigkeit, die Lebensdauer der Pneumococcen zu verlängern, zu prüfen. Die Versuche wurden in der Hauptsache im Laboratorium des Hospitals des Rockefeller-Instituts ausgeführt und gleichzeitig neben Eisenoxyden auch Eisenpentacyanverbindungen untersucht. Die Resultate waren für uns im Zusammenhang mit der vorhergehenden Arbeit sehr wichtig, da sie gleichzeitig unsere Ansicht bezüglich der Bedeutung der Eisenoxydkatalase bestärken konnten.

### *Ausführung der Versuche*

Pneumococcenkulturen, welche in Bouillon kräftiges Wachstum zeigen, werden (je 5 und 10 ccm) sowohl in Reagenzröhrchen als auch in Erlenmeyerkölbchen übertragen. In den ersteren kommen sie mit Luft wenig in Berührung,

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<sup>1</sup> Daß die Pneumococcen in Gegenwart von Blut große Lebensdauer besitzen, beruht nicht nur auf die Gegenwart von Katalase, sondern hängt auch mit der Verzögerung der Autolyse durch das Serum zusammen. Der letztere Effekt wird aber erst bemerkbar, wenn verhältnismäßig große Serummengen vorhanden sind.

während sie in flachen Schichten in den Kölbchen rasch aussterben. Die zu untersuchenden Substanzen werden unter der üblichen bakteriologischen Technik in die Gefäße eingetragen und im Dunkelraum bei Zimmertemperatur stehengelassen. Von Zeit zu Zeit werden Proben entnommen und auf Blutagarplatten gestrichen.

TABELLE I

*Kultur: Avirulenter Pneumococcus (Stamm R vom Typ II erhalten) in gewöhnlicher Bouillon*

Behandlung	Kulturen in den folgenden Zeiten (in Tagen) geprüft				
	9	12	16	24	40
10 ccm in 250 ccm Erlenmeyerkölbchen.....	—	—	—	—	—
5 „ im Reagenzrohr.....	+	+	+	—	—
10 „ in Erlenmeyer + 150 mg aktives Eisenoxyd.....	+	+	+	+	+
10 ccm in Erlenmeyer + 30 mg aktives Eisenoxyd.....	+	—	—	—	—
10 ccm in Erlenmeyer + 150 mg inaktives Eisenoxyd.....	+	+	—	—	—
10 ccm in Erlenmeyer + 30 mg inaktives Eisenoxyd.....	+	—	—	—	—
10 ccm in Erlenmeyer + 5 mg Ferro-aquosalz.....	+	+	+	+	+
10 ccm in Erlenmeyer + 500 mg $\text{Fe}_2\text{O}_3$ aus Eisencarbonyl.....	+	+	+	+	—

TABELLE II

*Virulenter Pneumococcus (Type II) in Bouillon*

Behandlung	Anzahl in Tagen, nach welchen die Kulturen geprüft wurden			
	10	20	30	40
10 ccm in Erlenmeyer.....	—	—	—	—
10 „ „ „ + 500 mg $\text{Fe}_2\text{O}_3$ aus Eisencarbonyl.....	+	+	±	—
10 ccm in Erlenmeyer + 150 mg $\text{Fe}_2\text{O}_3$ aus Eisencarbonyl.....	+	±	—	—
10 ccm in Reagenzröhrchen unter Vaseline...	+	+	+	+

Mit virulenten Pneumococcuskulturen waren die Resultate nicht so charakteristisch, da die Zellen durch Autolyse zugrunde gingen.

Die Resultate sind in der Tabelle III ersichtlich.

*Webster* und *Baudisch*<sup>1</sup> hatten gefunden daß Natriumpentacyano-aquoferroat als  $\alpha$ -Faktor wirkt, während das Natriumpentacyano-aminoferroat keinen Einfluß ausübt. Chemisch ist in verdünnter, wässriger Lösung kaum ein Unterschied in der Katalasewirkung der beiden Verbindungen zu bemerken, wie *Baudisch* und *Davidson*<sup>2</sup> festgestellt hatten. Die *biologische* Methode der Katalasewirkung

TABELLE III

Nährmedium	Größe des Wachstums auf Blutagar in der folgenden Zeit (in Tagen)					
	4	6	8	12	14	23
Bouillon (B).....	++++	+	0	0	0	0
B Blut (Verdünnung $10^{-3}$ )..	++++	++++	++++	++++	++++	++++
B Ferroaquosalz (Verdünnung $10^{-3}$ ).....	++++	++++	+++	+++	+	0
B Ferroaquosalz (Verdünnung $10^{-4}$ ).....	++++	++	0	0	0	0
B Ferroaquosalz (Verdünnung $10^{-5}$ ).....	++++	++	0	0	0	0
B Ferroaquosalz (Verdünnung $10^{-6}$ ).....	++++	+	0	0	0	0
B Ferroaminosalz (Verdünnung $10^{-3}$ ).....	0	0	0	0	0	0
B Ferroaminosalz (Verdünnung $10^{-4}$ ).....	++++	++	0	0	0	0
B Ferroaminosalz (Verdünnung $10^{-5}$ ).....	++++	+	0	0	0	0
B Ferroaminosalz (Verdünnung $10^{-6}$ ).....	++++	+	0	0	0	0

++++ bedeutet sehr gutes Wachstum auf Blutagar.

+++ bedeutet gutes Wachstum auf Blutagar.

++ bedeutet schwaches Wachstum auf Blutagar.

+ bedeutet Wachstum einzelner Kolonien auf Blutagar.

0 bedeutet kein Wachstum auf Blutagar.

konnte unter Umständen viel empfindlicher sein, und es wurden deshalb Versuche mit Pneumococcen ausgeführt.

<sup>1</sup> J. Exp. Med. 42, 473, 1925; siehe auch *Janet M. Bourn*, J. of Inf. Diseases 41, 294, 1927.

<sup>2</sup> Arch. of Intern. Med. 40, 496, 1927.

Je 5 ccm gewöhnliche Bouillon wurden in Reagenzgläsern verteilt und mit je 0,1 ccm einer 12 Stunden alten Kultur (avirulenter *Pneumococcus*) (Stamm R vom Typ II erhalten) geimpft. Die zu untersuchenden Salze wurden steril hinzugefügt und die Röhrchen erst 24 Stunden bei 37° und dann bei Zimmertemperatur im Dunkeln stehengelassen. Von Zeit zu Zeit wurden Proben entnommen und auf Blutagar gestrichen.

Die Versuche zeigen, daß das Ferroaquosalz selbst in großer Verdünnung einen günstigen Einfluß auf die Lebensdauer der *Pneumococci* ausüben. Ferroaminosalz hingegen verhält sich negativ und wirkt eher als Gift. Über die besonderen Eigenschaften des Ferroaquosalzes haben wir in mehreren Arbeiten ausführlich berichtet und gezeigt, daß die eine freie Valenz zu mannigfaltiger Wirkung fähig ist. Die koordinative Bindung von Sauerstoff oder von Wasserstoffsuperoxyd interessiert uns hier am meisten, weil sie mit der Katalasewirkung jener Salze in engster Beziehung steht.<sup>1</sup> Die von dem einen von uns beschriebenen Hemmungsreaktionen<sup>1</sup> des Ferroaquosalzes gibt uns ein Mittel in die Hand, um die Affinität chemischer Verbindungen zum zweiwertigen komplexen Eisen im Ferroaquosalz zu bestimmen. Verbindungen, welche starke Affinität besitzen (NH, SH, HCN, NO, CO u. a. m.) blockieren die freie Valenz durch Eintritt in den Komplex. Es ist daher leicht ersichtlich, daß das Ferroaquosalz, was seine katalatische Wirkung anbetrifft, leicht vergiftet werden kann. Wir haben gezeigt, daß es physiologisch wichtige Verbindungen gibt, welche stark blockierend wirken und somit ist es kein Wunder, daß die Versuche mit Ferroaquosalz in Bouillonkulturen von einigen Forschern nicht wiederholt werden konnten.<sup>2</sup> Um Ferroaquosalz erfolgreich als  $\alpha$ -Faktor anwenden zu können, muß man seine Katalaseaktivität in dem Kulturmedium zuerst prüfen, um sicher zu sein, daß nicht Eiweißspaltungsprodukte, vor allem Cystein oder Glutathion, das wirksame Eisen vergiftet haben. Ein biologischer Versuch mit *Pneumococci*, wie wir ihn hier demonstriert haben, dürfte sich für eine derartige Prüfung gut eignen. Auf dieser neuen Erkenntnis konnten wir die früheren Versuche von *Webster* und *Baudisch* ohne Schwierigkeiten reproduzieren.

Wie aus der Tabelle I und II zu ersehen ist, sind aktives  $\text{Fe}_2\text{O}_3$  und  $\text{Fe}_3\text{O}_4$  aus Eisencarbonyl ebenfalls wirksame Stoffe, um die Lebens-

<sup>1</sup> Diese Zeitschr. 232, 35, 1931.

<sup>2</sup> Zeitschr. f. exper. Med. 65, 805, 1929.

dauer des Pneumococcus zu verlängern. Beide Oxyde zeigten relativ starke Katalasewirkung. Bezüglich der Stabilität derartig aktiver Eisenoxyde und über ihre Empfindlichkeit gegenüber Katalasegiften haben wir bis jetzt noch keine Erfahrung. Die Fortsetzung der Versuche über die *biologische* Prüfung der Katalasewirkung einfacher anorganischer Verbindungen sollte für die Bakteriologie nutzbringend sein, da es diesbezüglich an empfindlichen chemischen und physikalischen Methoden mangelt.



**FACTORS AFFECTING THE YIELD OF SPECIFIC ENZYME  
IN CULTURES OF THE BACILLUS DECOMPOSING THE  
CAPSULAR POLYSACCHARIDE OF TYPE III  
PNEUMOCOCCUS**

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In a previous paper (1), a method has been described for the isolation from complex organic material of a microorganism capable of decomposing the capsular polysaccharide of Type III Pneumococcus. The principle of this method consists in the use of a selective medium containing as sole source of carbon a small amount of Type III capsular polysaccharide. In this medium, many of the irrelevant bacteria are rapidly eliminated, and only those organisms are selectively stimulated which are potentially capable of decomposing the polysaccharide in question.

Although the medium used had been originally devised for the isolation of the specific organism, it was found that, from cultures of the organism in this medium, an enzyme could be obtained which was capable of decomposing the capsular polysaccharide in the absence of living cells or even cell debris.

Although the synthetic medium—or modifications of it—has lent itself on several occasions to the isolation of organisms adapted to specific purposes, the fact was soon recognized that it was far from being satisfactory for the production of the enzyme. The occasional failure to obtain any active enzyme from cultures of the “S III bacillus” in the original, synthetic medium, led us to investigate the possibility of developing a more dependable technique.

The present paper is concerned with practical methods of production, purification, and concentration of the enzyme, with special regard to the composition of the medium, conditions of incubation of the culture, methods of extraction of the enzyme, and the influence of these factors on the potency and primary toxicity of the preparations.



## EXPERIMENTAL

*Methods*

1. *The Organism.*—The culture previously described (1) has been carried in the synthetic mineral medium containing the capsular polysaccharide (0.002 per cent) as sole source of energy. For each experiment, the culture is transferred to a medium consisting of a 1 per cent casein hydrolysate (at pH 7.0) in which it grows abundantly. The bacilli are separated from the 18 hour old culture by centrifugalization and washed once in saline before being used for inoculation of the test medium.

2. *Medium.*—The capsular polysaccharide was prepared from a strain of Type III Pneumococcus by the method previously described (2). The yeast extract used in these experiments is a commercial preparation available under the name "Difco yeast extract." The casein hydrolysate is also a commercial preparation known as "Tryptophane broth."

The soil extract was prepared by heating garden soil with an equal weight of 0.1 per cent sodium carbonate solution for 30 minutes at 15 lbs. pressure.

3. *Serological Method for Following the Decomposition of the Specific Polysaccharide.*—The presence or absence of the specific substance in the test fluid was determined by the precipitin reaction.

0.5 cc. of the fluid to be tested was added to 0.2 cc. of Type III antiserum<sup>1</sup> and the mixture brought to a volume of 1 cc. by the addition of salt solution. Since the precipitation test gives a positive result with a concentration of specific substance as low as 1:5,000,000, the absence of a positive precipitin reaction was interpreted as evidence of complete decomposition of the specific polysaccharide.

4. *Method of Enzyme Titration.*—As previously shown (1), there exists a definite quantitative relationship between the amount of specific polysaccharide decomposed and the quantity of enzyme used. The existence of this relation makes it possible to titrate the potency of any given enzyme preparation by the following method.

Varying amounts of the preparation to be tested are added to 1 cc. of a standard solution of Type III capsular polysaccharide (0.001 per cent concentration) adjusted at pH 7.0 with phosphate buffer. The mixtures are made up to 1.5 cc. and incubated for 18 hours at 37°C. in the presence of toluene. They are then tested for the presence of specific polysaccharide by the serological method.

The smallest amount of enzyme capable of completely decomposing the standard amount of capsular substance (0.01 mg.) under these conditions is a measure of the potency of the preparation. It has been found convenient to express the potency of any given preparation in terms of units. A unit of enzyme is defined

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<sup>1</sup> The Type III antipneumococcus serum used in these experiments was obtained through the courtesy of Dr. A. B. Wadsworth, Director of the Division of Laboratories of New York State Department of Health.

as one hundred times the smallest amount which will bring about the complete decomposition of 0.01 mg. of the purified specific capsular polysaccharide in 18 hours at 37°C. The number of units per cc. of preparation is the reciprocal of this smallest amount (expressed in cc.).

For instance, if it takes 0.001 cc. of a certain enzyme preparation to decompose the standard amount of capsular polysaccharide, the potency of the preparation will be  $\frac{1}{0.001 \times 100} = 10$  units per 1 cc.

It has been reported previously (1) that the S III bacillus grows abundantly on several common bacteriological media such as nutrient broth, peptone solution, casein hydrolysate, etc. The following experiment deals with attempts to determine whether the organism is capable of producing the specific enzyme in media not containing the capsular polysaccharide.

*Effect of the Presence or Absence of the Capsular Polysaccharide in the Medium on the Production of the Specific Enzyme by the S III Bacillus*

*Experiment 1.*—Three tubes each containing 5 cc. of casein hydrolysate medium, a fourth tube containing 1 per cent of galactose in 5 cc. of basic mineral medium, and a fifth tube containing 0.005 per cent of capsular polysaccharide in 5 cc. of basic mineral medium, were inoculated with a young culture of the S III bacillus, and the cultures incubated at 37°C. for 48 hours. The cells from two of the cultures in casein hydrolysate were separated by centrifugalization and resuspended in 5 cc. of saline; they were then broken up in one case by repeated freezing and thawing, in the other by extraction at 37°C. for 2 days in the presence of toluene.

The whole cultures and the cell extracts were then titrated for the presence of active enzyme; the results of the titrations are given in Table I.

The results of this experiment indicate that no appreciable amount of specific enzyme was formed when the capsular polysaccharide was not present in the medium. It is worth stating here that the growth was very scant in the specific substance medium although it was fairly abundant in the casein or galactose medium. This shows that the amount of growth is no measure of enzyme production.

The significance of this experiment becomes greater when it is realized that similar tests have been made with over 50 different media including simple and complex saccharides, organic acids, alcohols, pep-

tones, etc., in dilutions ranging from 0.01 to 2 per cent. Apart from the specific polysaccharide only one other substance caused the production of the specific enzyme by the S III bacillus, and this was the aldobionic acid derived from the capsular polysaccharide of Type III *Pneumococcus*. When, however, another aldobionic acid was used, *i.e.* that derived from gum arabic,<sup>2</sup> no growth, and, therefore no enzyme, could be obtained in the synthetic medium.

The conclusion seems justified therefore that, under the conditions of this experiment, the production of the specific enzyme by the S III

TABLE I

*Effect of the Presence or Absence of the Capsular Polysaccharide on the Formation of the Specific Enzyme by the S III Bacillus*

Nature of the preparation	Titration of the enzymatic potency of the cultures		
	Specific precipitin reaction given by mixtures of a standard amount of capsular polysaccharide with the following amounts of the different preparations (in cc.)		
	0.5	0.2	0.1
Whole culture in 0.005 per cent specific substance .....	—	—	—
Whole culture in 1 per cent galactose .....	++++	++++	++++
Culture in 1 per cent casein hydrolysate .....			
(a) Whole culture .....	++++	++++	++++
(b) Frozen and thawed cells .....	++++	++++	++++
(c) Cells extracted with toluene .....	++++	++++	++++

*Plus* signs indicate amount of precipitate formed in Type III antiserum.

*Minus* signs indicate no precipitation in Type III antiserum (complete decomposition of the specific polysaccharide).

bacillus is conditioned by the presence in the medium of the capsular polysaccharide itself, or of the aldobionic acid derived from it.

Since the secretion of the specific enzyme seems to be a response to the presence of the capsular polysaccharide in the medium, it was of interest to establish whether the amount of enzyme formed would bear any relation to the concentration of the specific substrate in the medium.

<sup>2</sup> The sample of aldobionic acid from gum arabic was obtained through the courtesy of Dr. M. Heidelberger of the Presbyterian Hospital, New York.

*Effect of the Concentration of Specific Substance in the Synthetic Medium  
on the Yield of Specific Enzyme*

*Experiment 2.*—A basic medium was prepared of the following composition.

0.2 per cent $K_2HPO_4$	} . . . . . pH 7.0
0.1 per cent $(NH_4)_2SO_4$	
5 per cent soil extract	
Tap water	

This medium was distributed in 30 cc. amounts in eight Erlenmeyer flasks (250 cc. capacity). To these flasks were added different amounts of specific substance to give final concentrations ranging from 0.01 per cent to 0.5 per cent. The media were inoculated with a young culture of the S III bacillus and incubated at 37°C. After incubation for 1 week, the cultures were filtered and the filtrates titrated for enzyme activity. The results of the titrations are given in Table II, and also the time required for complete decomposition of the capsular polysaccharide in the original culture.

The results of this experiment establish the fact that in the synthetic medium growth of the S III bacillus is inhibited by concentrations of capsular polysaccharide higher than 0.3 to 0.4 per cent. They also indicate that, whereas the yields of enzyme rapidly increase with increasing concentrations of specific substrate in the medium within the range of 0.01 per cent to 0.1 per cent, the yields begin to decrease when the concentration exceeds 0.1 per cent. This is an unexpected finding, and one is at first tempted to explain it in terms of the preceding observation, namely, that high concentrations of capsular polysaccharide are toxic to the organisms and the toxicity in some way may decrease the yield of enzyme. There are so far no facts to rule out this hypothesis, but another explanation suggests itself when the culture is followed microscopically from day to day. It has already been mentioned that the cells undergo autolysis very rapidly in the synthetic culture medium. In fact, in the present experiment, the cultures exhibited cell disruption and spore formation within 24 hours after inoculation, and, on the 3rd day, the spores far outnumbered the visible cells. It is known that, under such conditions, the enzyme is liberated into the medium. Now, the results given in Table II show that, at the higher concentrations of specific substrate, it took 3 to 4 days before the capsular polysaccharide was completely decomposed. We may, therefore, assume that for 2 days at least, free enzyme and undecomposed

specific substance were simultaneously present in the medium. Under these conditions there occurred a decomposition of a portion of the capsular polysaccharide by the free enzyme and this decomposition was unrelated to the metabolic activity of the organism. During the process, inactivation of the enzyme liberated from the autolyzed cells occurred. The low yields of enzyme may, therefore, be due to these two causes: (a) loss of capsular polysaccharide and a resulting decreased amount of growth, (b) inactivation of the enzyme already

TABLE II

*Effect of the Concentration of Capsular Polysaccharide in the Medium on the Yield of Enzyme from Cultures of the S III Bacillus*

Medium containing capsular polysaccharide	Time required for complete decomposition of capsular polysaccharide in the original culture	Titration of enzymatic potency of culture filtrates					
		Specific precipitin reaction given by mixtures of a standard amount of capsular polysaccharide and the following amounts of filtrate of the different cultures (in cc.)					
		0.5	0.2	0.1	0.05	0.02	0.01
per cent	days						
0.5	No growth	x	x	x	x	x	x
0.4	No growth	x	x	x	x	x	x
0.3	4	—	—	+	+++	+++	+++
0.2	4	—	—	—	+	+++	+++
0.1	3	—	—	—	—	++	+++
0.05	3	—	—	—	+	+++	+++
0.02	2	—	—	+	+++	+++	+++
0.01	2	—	+	+++	+++	+++	+++

x indicates not done.

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum (complete decomposition of the specific polysaccharide).

formed. If such were the case, a possible solution of the problem would be to devise a medium in which the growth would be so rapid that complete decomposition of the polysaccharide would be obtained within 24 hours, while at the same time the rate of autolysis would be decreased so that the release of enzyme into the medium would be retarded.

It is not necessary to report here all the modifications of—and additions to—the synthetic medium which were tested to determine this

effect. Only those changes in procedure will be reported which have given a solution of the problem; *i.e.*, addition of yeast extract to the medium and incubation of the cultures under conditions of increased aeration.

*Effect of Yeast Extract on the Yield of Specific Enzyme*

*Experiment 3.*—To three Erlenmeyer flasks (250 cc. capacity) each containing 50 cc. of the basic mineral medium, yeast extract or capsular polysaccharide, or

TABLE III

*Effect on the Yield of Enzyme of Addition of Yeast Extract to the Synthetic Medium*

Nature of the medium	Time required for complete decomposition of polysaccharide in original culture medium	Titration of enzymatic potency of culture filtrates					
		Specific precipitin reaction given by mixtures of a standard amount of capsular polysaccharide and indicated amounts of filtrate of the different preparations (in cc.)					
		0.5	0.2	0.1	0.05	0.02	0.01
	<i>hrs.</i>						
0.1 per cent yeast extract.....	x	++++	++++	++++	++++	++++	++++
0.1 per cent specific substance.....	72	—	—	++	++++	++++	++++
0.1 per cent yeast extract + 0.1 per cent specific substance.....	24	—	—	—	—	—	+

x indicates no capsular polysaccharide.

Plus signs indicate amount of precipitate formed in Type III antiserum.

Minus signs indicate no precipitation in Type III antiserum (complete decomposition of the specific polysaccharide).

both, were added in the following concentrations respectively: (a) 0.1 per cent yeast extract, (b) 0.1 per cent specific polysaccharide, (c) 0.1 per cent yeast extract + 0.1 per cent specific polysaccharide. The media were inoculated as usual and incubated at 37°C. for 5 days.

The three cultures were separately filtered through Berkefeld filters (N) and the respective filtrates were tested for enzyme action. The readings of the titrations are reported in Table III.

In the culture containing only the capsular polysaccharide in the mineral medium, the specific substrate was decomposed in 3 days,

whereas the same result was obtained within 24 hours in the culture containing both polysaccharide and yeast extract.

Microscopic examinations revealed interesting differences between the three cultures. After 3 days incubation, only long, well formed bacilli were to be seen in the culture containing both capsular polysaccharide and yeast extract, whereas after 24 hours incubation, the other two cultures exhibited only spores and cell debris.

These results as well as the titrations of the filtrates given in Table III clearly show that the addition of 0.1 per cent yeast extract to the synthetic medium increases the rate of decomposition of the capsular polysaccharide by the S III bacillus, retards the process of autolysis, and increases tenfold the yield of specific enzyme from a definite amount of specific substrate. It is worth noting again that, although good growth was obtained in the medium containing the yeast extract alone, no specific enzyme could be detected, confirming once more the results obtained in Experiment 1.

Later experiments have shown that even smaller concentrations of yeast extract serve to bring about the same results. However, the results become less reliable when the concentration of yeast extract is less than 0.03 per cent.

It has been mentioned previously that the S III bacillus is an obligate aerobe. In fact, the aerobic character of this organism is so pronounced that the decomposition of the capsular polysaccharide is much slower when the culture is incubated in a test tube (5 cc. of medium per tube) than when it is exposed in shallow layers in a flask. The following experiment establishes the effect of the conditions of aeration on the rate of decomposition of the specific substance by the S III bacillus, and on the yield of specific enzyme from the cultures.

#### *The Effect of Aeration on the Yield of Specific Enzyme*

*Experiment 4.*—The medium used was the basic mineral medium containing in addition 0.03 per cent yeast extract and 0.1 per cent specific substance. It was distributed in 15 cc. amounts into two Erlenmeyer flasks (300 cc. capacity) and two large test tubes of 3 cm. diameter. This provided conditions of aeration such that the surface area of the culture medium exposed to air was about 50 cm.<sup>2</sup> in the flasks, and 5 cm.<sup>2</sup> in the tubes. Flasks and tubes were seeded with a heavy inoculum of a young culture of the S III bacillus.

The specific substance was completely decomposed in 1 day in the flasks and in

2 days in the tubes. On the 3rd day, the culture filtrates were titrated for enzyme activity. The results of the titrations are given in Table IV.

Experiment 4 brings out the very striking facts that (1) as the surface area of the culture medium exposed to air was increased from 5 cm.<sup>2</sup> to 50 cm.<sup>2</sup>, the rate of decomposition of the capsular polysaccharide was doubled and (2) the yield of specific enzyme in the filtrate was increased tenfold. Although quantitative studies on the amount of growth will not be described here, it can be stated that, roughly speaking, the growth was also ten times heavier in the flasks than in the

TABLE IV

*Effect of Aeration on the Yield of Specific Enzyme from Cultures of the S III Bacillus Grown in the Presence of Capsular Polysaccharide*

Surface area of the culture medium exposed to air (approximate)	Time required for complete decomposition of the polysaccharide in the original culture	Titration of enzymatic potency of culture filtrates				
		Specific precipitin reaction given by mixtures of a standard amount of capsular polysaccharide and the indicated amount of culture filtrate (in cc.)				
		0.01	0.05	0.03	0.01	0.005
cm. <sup>2</sup>	hrs.					
50	24	—	—	—	+	+++
50	24	—	—	—	—	+++
5	48	+	+++	+++	+++	+++
5	48	+	++	+++	+++	+++

*Plus* signs indicate amount of precipitate formed in Type III antiserum.

*Minus* signs indicate no precipitation in Type III antiserum (complete decomposition of the specific polysaccharide).

tubes. The emphasis to be placed on incubation of the cultures in shallow layers becomes still greater when one realizes that the conditions obtaining in the tubes would have been considered sufficiently aerobic for most common organisms, since the depth of culture fluid was less than 4 cm. and the medium itself did not have any appreciable reducing power.

The S III bacillus was originally isolated from a mixed culture growing at 37°C. However, the organism can also decompose the capsular polysaccharide at lower temperatures. The purpose of the following experiment was to establish the effect of incubation temperature on the



rate of decomposition of the specific substrate and on the yield of specific enzyme.

*Effect of Temperature of Incubation on the Rate of Decomposition of the Capsular Polysaccharide and on the Yield of Specific Enzyme from the Culture*

*Experiment 5.*—The medium used contained 0.1 per cent yeast extract and 0.1 per cent specific polysaccharide. It was distributed in 100 cc. amounts into three Blake bottles which, after inoculation with a young culture of the S III bacillus, were incubated in the horizontal position so as to insure suitable conditions of

TABLE V

*Effect of Temperature of Incubation on the Rate of Decomposition of Capsular Polysaccharide by the S III Bacillus and on the Yield of Specific Enzyme*

Incubation temperature	Time required for complete decomposition of the capsular polysaccharide in the original culture medium	Titration of enzymatic potency of culture filtrates			
		Specific precipitin test of mixtures of standard amount of capsular polysaccharide and indicated amounts of culture filtrate (in cc.)			
		0.05	0.02	0.01	0.005
	<i>hrs.</i>				
37°C.....	24	—	—	+	++++
30°C.....	48	—	—	—	+
Room temperature.....	120	—	++	++++	++++

*Plus* signs indicate amount of precipitate formed in Type III antiserum.

*Minus* signs indicate no precipitation in Type III antiserum (complete decomposition of the specific polysaccharide).

aeration. The temperatures of incubation were respectively 37°C., 30°C., and room temperature. After 2 weeks incubation,<sup>3</sup> the cultures were filtered through Berkefeld filters (N) and the filtrates titrated for enzymatic activity (Table V).

It appears from the results (Table V) that, although the organisms grow more rapidly at 37°C. than at 30°C. the yield of enzyme is higher at the lower temperature. Room temperature is unfavorable, both from the point of view of rate of decomposition and yield of specific enzyme.

<sup>3</sup> The longer period of incubation in this experiment was necessary for the completion of autolysis in the culture at room temperature.

*Effect of Length of Incubation and Filtration of the Culture on the Yield of Enzyme*

It has been repeatedly mentioned that, as the result of autolysis, the specific enzyme is rapidly released into the medium. However, experience has shown that the greatest yield of enzyme is not obtained immediately after complete decomposition of the capsular polysaccharide, but a few days later. This point is now the object of further analysis and will not be discussed here. In routine procedure, the cultures are incubated for 7 to 14 days before filtration is carried out.

It is of obvious advantage to have the enzyme preparations as clear and cell-free as possible. For this purpose, the autolysates are filtered through Berkefeld candles (N) before being tested. Titrations of enzyme potency of the preparations before and after filtration have always shown that there is no loss of activity during the process. This indicates that the enzyme is not adsorbed on the candle, at least not at the pH (7.0) at which filtration is carried out.

*Concentration of the Enzyme Preparations*

With the routine procedure now in use in this laboratory, the culture filtrate has an enzymatic activity which varies from 1 to 1.2 units per cc. Attempts were made to concentrate the preparations by the following methods: (a) specific adsorption, (b) precipitation with alcohol and acetone, (c) distillation under reduced pressure, (d) ultrafiltration.

The specific enzyme can be adsorbed with small amounts of kaolin, alumina gels, and bone charcoal, provided the adsorption experiments are carried out at alkaline pH. Unfortunately it has not yet been possible to recover the enzyme from the adsorbent and consequently this method has not as yet proved serviceable.

Precipitation with alcohol and acetone has been only partly successful since it is accompanied by the rapid inactivation of the enzyme (at room temperature at least).

Good results have been obtained by concentration under reduced pressure. During vacuum distillation, the water bath was maintained at a temperature below 35°C., and the vacuum was obtained with an oil pump. Under these conditions, the preparations could be con-

centrated tenfold without any inactivation of the enzyme. The whole activity could be quantitatively recovered in the concentrate.

Equally successful results have been obtained by ultrafiltration; the optimum formula for the preparation of the membrane on alundum thimbles is as follows:

	gm.
Soluble cotton.....	6
Glacial acetic acid.....	122
Anhydride potassium carbonate.....	2

With this membrane, the enzyme is retained in the ultrafilter and the filtrate is completely inactive. The ultrafiltration method has been adopted in preference to vacuum distillation. When properly carried out, it requires less care and has the special advantage of eliminating in the filtrate a considerable amount of the irrelevant material.

#### *Effect of the Methods of Preparation of the Enzyme on Primary Toxicity*

The protective action of the specific enzyme on Type III pneumococcus infections in mice has been described in a previous paper (3). At that time, no primary toxicity of the preparations was observed in the treated animals. Up to 1.5 cc. of the enzyme preparations were injected intraperitoneally into normal mice without any apparent discomfort to the animals. The enzyme preparations then used had been prepared by growing the S III bacillus in the mineral medium containing the capsular polysaccharide as sole source of carbon.

Following the use of yeast extract in the medium, it was observed that the intraperitoneal injection of the enzyme into mice resulted in peritoneal irritation. The same preparation, when injected intravenously into normal rabbits, also exhibited marked toxicity—sudden rise in temperature—and at times death within 2 to 3 minutes after injection.

Two factors have been found to have a profound influence on the development of primary toxicity. Although yeast extract *per se* is not toxic, cultures in a medium containing this substance prove to be toxic. As a result of this observation, the amount of yeast extract in

the medium has been reduced to the lowest possible concentration compatible with good yields of enzyme; as already mentioned, this minimum concentration is 0.03 per cent.

On several occasions, toluene had been added to the culture medium after growth had developed so as to obtain a more complete lysis of the cells and more rapid release of the enzyme. All the preparations thus treated were highly toxic, even though every precaution had been taken to remove as completely as possible all traces of toluene before use in animals. It seems likely that toluene causes solution of some toxic cellular products which are otherwise retained by the Berkefeld filter.

### *Purification of the Enzyme Preparations*

When the enzyme preparations are obtained by the routine technique now in use in this laboratory, the primary toxicity for normal rabbits is so low that it can be detected only when large amounts are injected. However, attempts were made to further decrease the toxicity by some method of purification.

Dialysis failed since both the enzyme and the toxic principles remain in the dialysate. Specific absorption of the enzyme could not be used since, as already mentioned, the active enzyme could not be recovered from the adsorbent.

A partial solution of the problem was found in devising conditions under which some of the toxic principles would be adsorbed and the active enzyme itself remain in solution. These conditions are fulfilled when a proper amount of aluminum gel (Willstätter's Preparation C) is added to the enzyme previously adjusted to pH 5.5. After a few minutes contact, the supernatant is separated from the gel by centrifugalization. The injection into normal rabbits of this supernatant, in which the specific enzyme is still present, does not affect the temperature and behavior of the animals any more than does a similar injection of normal saline. This technique has always given satisfactory results even when carried out on those preparations which were originally the most toxic. Further studies on the use of these purified preparations in rabbits are presented in the accompanying paper (4).

## DISCUSSION

As already stated, the object of the studies described in this paper was of a purely practical nature; *i.e.*, the development of a reliable technique for the preparation of a potent, purified enzyme, capable of decomposing the capsular polysaccharide of Type III Pneumococcus. However, the results have also brought out a few points of interest in the understanding of enzyme production by microorganisms in general.

First, it must be appreciated that the final yield of active enzyme may be no expression of the total amount of enzyme produced by the culture. Several cases have been described in this paper in which both the living microorganism and the enzyme already free in the medium compete for the specific substrate. It appears that conditions may prevail such that the specific substrate may be decomposed by the free enzyme without the microorganism benefiting thereby. This point must be considered in quantitative studies of enzyme production, especially when, as in this instance, the enzyme is quantitatively inactivated in the course of decomposition of the specific substrate.

The high yields of specific enzyme obtained by the addition of small amounts of yeast extract to the medium and by incubation in shallow layers have been traced to a more rapid decomposition of the capsular polysaccharide by the S III bacillus, and a decreased rate of autolysis. Such factors prevent the enzyme released into the medium from expending itself—as it were—on the capsular polysaccharide, thus leaving a larger part of the specific substance as utilizable source of energy for the growing microorganism. However, some more direct form of activation of enzyme production by the yeast extract and oxygen from the air cannot as yet be ruled out.

One cannot help being impressed by the tremendous stimulation of growth in all kinds of media under conditions of increased aeration. This stimulation always expresses itself in the synthesis of a much larger amount of bacterial protoplasm. In media containing the Type III capsular polysaccharide, increased aeration also results in a more rapid decomposition of this polysaccharide, and in larger yield of specific enzyme (Experiment 4).

Finally—and not least interesting—is the fact that, under the con-

ditions described in Experiment 1, the presence of specific enzyme could be detected only when the medium contained the capsular polysaccharide itself, or the aldobionic acid derived from it. Suggestions of a similar nature are often found in the microbiological literature, but they are the object of much controversy. The case described here seems the more significant since, as already mentioned in a previous paper, the capsular polysaccharide is not an especially favorable source of energy for the S III bacillus, but is attacked only when no other available nutrient is present in the medium. Teleologically speaking, the secretion of the specific enzyme appears then as an emergency measure on the part of the bacterial cell otherwise deprived of growth energy.

#### SUMMARY

An improved method is described for the preparation, concentration, and purification of a bacterial enzyme capable of decomposing the capsular polysaccharide of Type III Pneumococcus.

The cultural conditions for the growth of the specific microorganism must be such that the capsular polysaccharide is completely decomposed before any appreciable amount of free enzyme is released into the medium. This reduces to a minimum the decomposition of the specific substrate by the free enzyme. As a result, a larger part of the specific substance remains as a source of energy for the growing microorganism and less enzyme is lost through inactivation during the course of decomposition of the specific substrate.

A marked stimulation of growth and of enzyme production occurs when small amounts of yeast extract are added to the medium and when the cultures are incubated under conditions of increased aeration.

Special emphasis is placed upon the fact that, thus far, appreciable amounts of the specific enzyme have been obtained only when the capsular polysaccharide itself, or the aldobionic acid derived from it, was present in the culture medium.

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## THE ACTION OF A SPECIFIC ENZYME UPON THE DERMAL INFECTION OF RABBITS WITH TYPE III PNEUMOCOCCUS

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Avery and Dubos (1-4) have described an enzyme of bacterial origin which possesses the property of specifically decomposing the capsular polysaccharide of Pneumococcus Type III. When injected together with the infecting organisms this enzyme protects mice against infection with Type III pneumococci and has a curative action when given after a generalized infection has been established.

The present paper deals with the curative action of this specific enzyme in the experimental disease brought about by infecting rabbits intradermally with a strain of Type III Pneumococcus of high virulence for these animals.

The characteristics of this experimental disease in rabbits have been previously described, especially with reference to Type I pneumococcus infections (5-8). The disease offers peculiar advantages for the study of the curative action of a specific agent since the lesion is visible and the course of the infection can easily be followed.

In order to produce the experimental disease with Type III pneumococci it is necessary to use a strain which is highly virulent for rabbits. In these experiments we have used a culture of Type III Pneumococcus designated "PH," a virulent strain previously described by Tillett (9) in his studies of the infectivity of Type III Pneumococcus for rabbits.

The usual strains of Type III Pneumococcus, even when freshly isolated from cases of lobar pneumonia, are not highly virulent for rabbits, and in most instances the symptoms are mild as compared with those resulting from infection with Type I pneumococci. If a sufficient number of organisms are used a fatal infection



can be brought about with some strains but the results are somewhat irregular. Similar experiences have also been reported by Tillett (9), and by Watson and Cooper (10). Occasionally, however, a strain is encountered which is highly virulent for rabbits. Such a strain was employed in these experiments.

#### EXPERIMENTAL

The methods of infecting the rabbits and of following the course of the disease differed in no essential from those described in previous studies.

*Pneumococcus Cultures.*—The Type III Pneumococcus used in these experiments was the rabbit-virulent strain described by Tillett (9) under the designation "PH." This was grown in rabbit blood broth and possessed a virulence for mice such that 0.000,000,01 cc. of an 18 hour culture, given intraperitoneally, sufficed to kill within 96 hours. The virulence for rabbits was maintained by frequent animal passage and was such that 0.000,01 cc., given intradermally, caused death or a protracted disease of severe character. With doses of 0.001 cc. only about 5 per cent of untreated animals ultimately recover.

*Infection.*—Healthy male rabbits of 1,800 to 2,200 gm. were selected, and the hair was removed from the abdominal and flank areas. The animals were then injected intradermally, at a site midway on the flank area, with 0.2 cc. of a dilution of the culture containing the desired number of organisms. In the experiments reported in this paper the infective amount was 0.001 cc. of an 18 hour blood broth culture.

*Enzyme Preparations.*—The enzyme preparations used in these experiments were, for the larger part, purified and concentrated by the method described by Dubos (4). For the purposes of the present study a unit of enzyme may be defined as one hundred times the smallest amount which will bring about the complete decomposition of 0.01 mg. of the purified specific capsular polysaccharide in 18 hours at 37°C. The exact method for its quantitative estimation has been previously described (4).

*Therapeutic Treatment.*—The desired amount of enzyme preparation was warmed to 37°C. and injected intravenously at a rate not greater than 1.5 cc. per minute. All preparations were isotonic and were adjusted approximately to pH 7.3.

The development of the lesion and the associated events differ in no essential from those observed in Type I pneumococcus infections, and may be summarized as follows:

After the intradermal injection of 0.001 cc. of an 18 hour culture of rabbit-virulent Type III Pneumococcus in the flank area of the rabbit, there is at first a latent period of 3 to 5 hours. Then, near the point of inoculation there appear signs of

early inflammation. Edema appears and spreads ventrally at a rate of 2 to 3 cm. an hour, carrying with it the infecting organisms. After 9 to 12 hours the edema has usually reached the ventral midline. More and more edema fluid collects in this area until the entire zone becomes tense and swollen. Within 20 to 28 hours a moderate degree of induration is present and this increases gradually until at 48 hours the involved tissue is quite firm. The lesion is marked by an orange-red color, which in the spreading lesion always develops subsequent to the passage of the edema fluid through a particular area. The local lesion resembles that induced with Type I *Pneumococcus* except that there is a greater amount of purpura and of frank hemorrhagic necrosis.

With the first developments in the local lesion, the temperature begins to rise and usually reaches a point well over 104°F. at 20 hours, then rises slowly to an average level of 106°F. This high temperature usually persists until the time of death or recovery.

*Pneumococci* usually appear in the circulating blood 15 to 22 hours after infection and the number rapidly increases. The severity of the disease at any one time appears to be directly related to the number of *pneumococci* in the blood stream.

Most of the untreated animals die on the 3rd or 4th day of the infection. A few rabbits recover, but only after a course of high temperature persisting for 8 or 9 days.

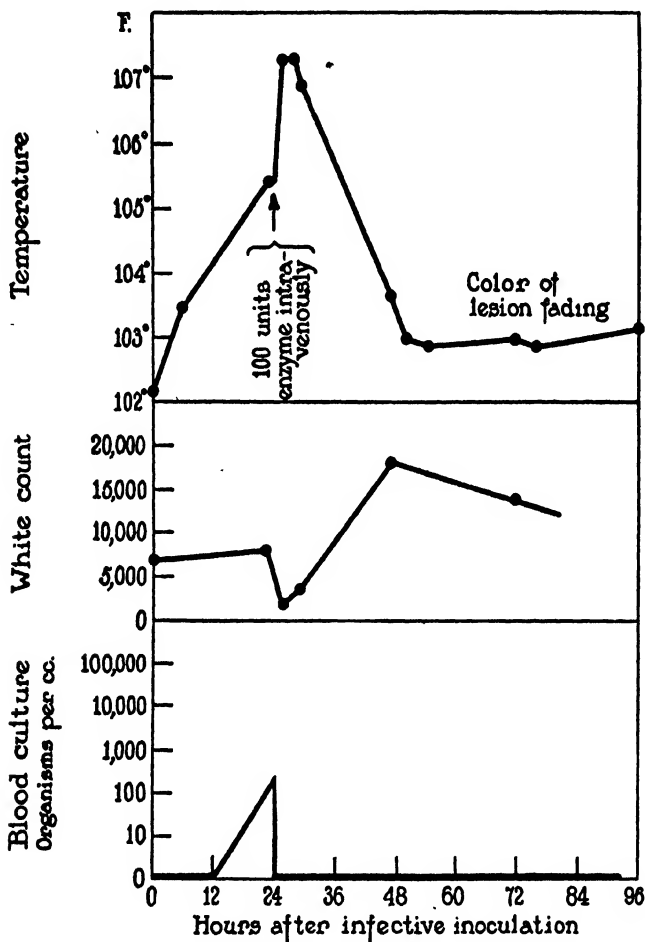
### *Course of Events Following the Administration of Enzyme*

At the beginning of these studies there existed no basis on which to judge the amounts of enzyme necessary to bring about recovery. It soon became apparent that by the injection of adequate amounts of the specific enzyme 24 hours after the infective inoculation, it was possible to bring about an early and complete cessation of the disease. The blood stream was freed of *pneumococci* and the organisms disappeared from the local lesion in the course of a few hours. Following the administration of the enzyme, the temperature at first became higher, but fell within 24 hours to normal levels. The local lesion failed to spread and soon showed signs of healing. A detailed account of the findings in treated rabbits follows.

*Temperature.*—The enzyme has usually been injected 24 hours following the infection, when the rectal temperature is usually 104°F. or higher. Following the administration of the enzyme the temperature rises abruptly, in many instances as high as 107°F. This rise appears to be slightly greater than that occurring after the administration of saline, serum, or vaccines. The peak is generally reached about 2 hours after the injection, but within 4 or 5 hours the temperature begins to

fall and is usually normal within 24 hours. Provided the amount of enzyme has been sufficient, the temperature remains low.

*Blood Cultures.*—Pneumococci may be present in the blood stream as early as 15 hours after the intradermal inoculation. At 24 hours the blood culture is

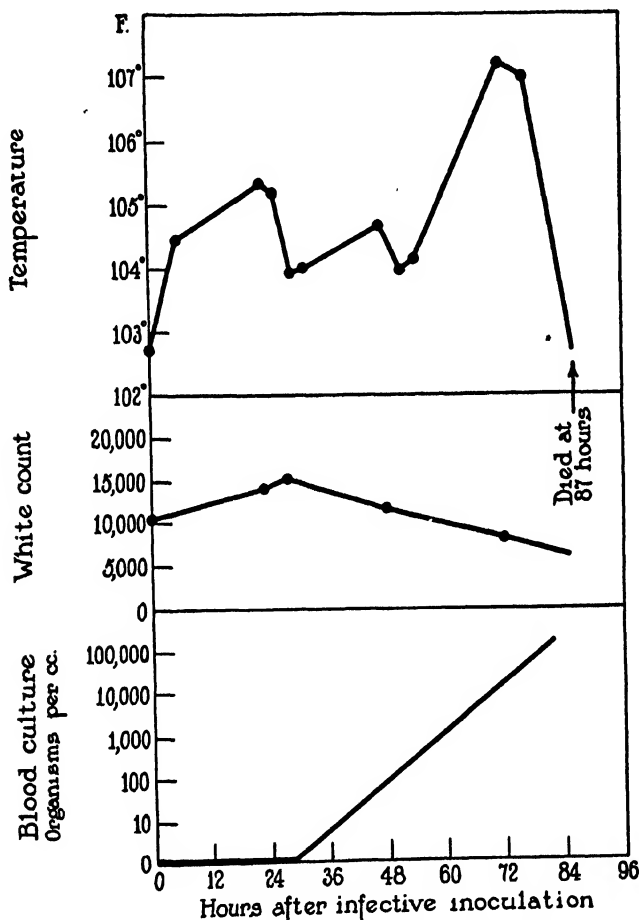


TEXT-FIG. 1. Chart of temperature, white count, and bacteriemia in the case of a rabbit treated with enzyme 24 hours after infective inoculation with Type III Pneumococcus.

usually positive, the number of organisms per cc. of the circulating blood varying from 5 to 10,000. Following the administration of suitable doses of the enzyme

the pneumococci promptly disappear from the blood, and there is no recurrence of bacteriemia.

*Local Lesion.*—At the time of treatment the local lesion shows a bright orange-red color and edema is a marked feature. More or less purpura is frequently in



TEXT-FIG. 2. Chart of temperature, white count, and bacteriemia in the case of a control rabbit infected intradermally with Type III Pneumococcus.

evidence, being more marked in the older and less edematous part of the lesion. The consistency of the lesion is still soft. After intravenous enzyme administration the intensity of the color of the lesion is increased, and in many instances a

large area becomes purpuric within a few hours. With the subsiding of the temperature following treatment the bright color of the lesion begins to fade. After 48 hours all of the non-purpuric areas have again taken on the normal color of the neighboring skin. Following the administration of the enzyme the lesion becomes much firmer and not only does not increase further in extent and magnitude, but actually diminishes. After several days most of the swelling has disappeared. The superficial necrotic material of the purpuric areas is not absorbed but sloughs, and the actual healing of the local lesion is slow and frequently requires many days. With the disappearance of the inflammatory color the surface of the lesion becomes much less glossy, and desquamation, which in other experiments has been associated with healing, makes its appearance.

Direct cultures of material aspirated from the lesion have usually shown viable pneumococci for several hours after treatment, but after 5 to 10 hours the cultures are sterile.

*Leukocytes.*—Immediately after treatment there is a marked diminution in the number of circulating white blood cells, but within a few hours, usually coincident with the fall of temperature, there occurs a definite increase in the number of these cells, frequently reaching as high as 40,000 per c.mm. The number gradually diminishes and usually reaches normal levels within a few days.

*Body Weight.*—Weight loss continues as long as the temperature remains above normal, but as soon as the critical changes in the course of the disease occurs, the loss in weight ceases and the original body weight is soon regained.

An illustration of the results obtained in one of a large series of enzyme-treated rabbits is shown in Text-fig. 1. For comparison a typical example of the course and fatal outcome of the infection in the untreated control animals is shown in Text-fig. 2.

The case illustrated by Text-fig. 1 is typical.

24 hours after infection the temperature was 105.4°F. and the blood culture showed 165 pneumococci per cc. of blood. At this time, 100 units of the specific enzyme were injected intravenously. The temperature rose to 107.3°F., but after 5 hours began to fall rapidly. The temperature continued to fall until at 48 hours it was within the normal range. Immediately following the injection of the enzyme the pneumococci disappeared from the blood and did not again make their appearance. The recovery was uneventful.

The control rabbit (Text-fig. 2) had a milder disease than the one which received the enzyme enjection, since 24 hours after infection no bacteria were present in the blood stream. Nevertheless, in the absence of treatment with enzyme, the severity of the disease increased, and the animal died in 87 hours.

The results obtained in the treatment of a large series of infected animals indicate that in cases with severe bacteriemia large quantities

of the enzyme are necessary to obtain successful results, while in animals having fewer organisms in the blood at the time of treatment smaller amounts of the enzyme are adequate.

In our most recent work, nineteen infected rabbits have been treated with large amounts of concentrated enzyme. Of these only one died. On the other hand, among thirty-eight similarly infected rabbits which were untreated, only two survived, and then only after a severe and protracted illness.

TABLE I  
*Specificity of the Action of the Enzyme*

Infections in each case intradermal. Dilutions were arranged so that the required inoculum was in each case contained in 0.2 cc. of broth. Each treated animal received a single injection of 100 units of enzyme (6 cc.) intravenously 24 hours after infective inoculation.

Amount of infective inoculum cc.	Type of Pneumococcus used for infection			
	Type III		Type I	
	Treated	Untreated	Treated	Untreated
0.001	S S S	D 88	—	—
0.000,01	—	D 62	—	—
0.000,001	—	—	D 20	D 44
0.000,000,1	—	—	D 23	—
0.000,000,01	—	—	—	D 112

S = survival of animal; in each instance the temperature had fallen to normal levels within 24 hours after treatment and remained within the normal range.

D = death of animal; the numeral indicates the number of hours elapsing before death.

— = not done.

### *The Specificity of the Curative Action of the Enzyme*

In the work of Avery and Dubos, previously referred to, it was shown that *Pneumococcus* Type III bacteriemia in mice could be checked by the intraperitoneal injection of the specific enzyme and that this action was specific for Type III *Pneumococcus*. It seemed desirable to investigate the specificity of the action of the enzyme in

this experimental disease in rabbits in which the focus of the infection is more localized and less accessible to therapeutic agents introduced by the intravenous route.

An experiment which illustrates the specificity of the action of the enzyme is summarized in Table I. Both Type III and Type I pneumococcus dermal infections were treated with similar amounts of enzyme, in each instance 24 hours after infective inoculation. Control

TABLE II  
*Inactivation of Enzyme by Heat*

Six rabbits were infected intradermally with 0.001 cc. of rabbit-virulent Pneumococcus Type III broth culture. Two animals received single intravenous injections of enzyme 24 hours after infective inoculation. Two others were given the same amounts of enzyme solution which had been heated at 70°C. for 30 minutes. The remaining two animals similarly infected but untreated served as controls.

Infected rabbits	Enzyme (Lot 2-5-29)	Results
A	Unheated	S
B	Unheated	S
C	Heated at 70°C., for 30 min.	D 38
D	Heated at 70°C., for 30 min.	D 42
E	No enzyme	D 52
F	No enzyme	D 168

S = survival of animal; in each instance treatment was followed by the disappearance of organisms from the blood stream and by a gradual fall of temperature to normal levels.

D = death of animal; the numeral indicates the number of hours elapsing before death.

cases show that the rabbits received at least 100 minimal fatal infective doses of pneumococci in the case of Type III, and ten and 100 minimal fatal infective doses, respectively, in the case of Type I.

In this experiment, of the three rabbits infected with Type III Pneumococcus and subsequently treated with a single injection of the specific enzyme, all recovered, while of the two rabbits infected with Type I Pneumococcus and similarly treated both died. The curative action of the enzyme in this experimental disease, as in the mouse infections, is type-specific.

### *Heat Inactivation of the Enzyme*

It has been shown previously that the activity of the enzyme, as measured by *in vitro* methods and by the protection test in mice, is destroyed by heat. The following experiment was designed to determine whether heating the enzyme also destroyed its curative action in rabbits.

Six rabbits were each infected intradermally with 100 minimal fatal doses (0.001 cc.) of the rabbit-virulent strain of Type III Pneumococcus. After 24 hours two of these animals were treated intravenously with 100 units of enzyme, and two others were given the same amount of enzyme which had been heated at 70°C. for 30 minutes. The two remaining animals were untreated and served as controls. The results of this experiment are summarized in Table II.

In this experiment the animals which received injections of active enzyme recovered promptly. The two rabbits which were given the heated enzyme preparation died, as did also the two control animals. These results demonstrate that the curative principle of the enzyme preparation is heat-labile.

### *Active Immunity Following Enzyme Treatment*

A considerable number of rabbits which have recovered following the administration of enzyme have later been reinfected by intradermal injections of pneumococci in order to determine whether or not active immunity had developed. In each case the injection was made in the same area in which the original infective inoculation had been given. The amount injected was 0.2 cc. of an 18 hour blood broth culture of the rabbit-virulent strain of Type III Pneumococcus. This is a massive infective dose, but previous studies (6) have shown this to be the most suitable dose for determining the presence of active immunity. The results obtained in a typical experiment are shown in Table III.

In the animals which had recovered promptly following enzyme injections given 24 to 48 hours after the original infection, no immunity could later be demonstrated. These animals reacted to the subsequent infection exactly like normal animals to an initial infection. On the other hand, in animals which had been originally treated with repeated small doses of enzyme, so that the disease was not arrested



for several days, a high degree of active resistance to subsequent infection was found to be present.

Apparently in the first group of animals the specific antigen was destroyed too early to permit of its functioning as an effective stimulus to antibody formation. These results are analogous to those obtained

TABLE III

*Active Immunity in Rabbits Following Recovery from Infection after Enzyme Injections*

Each animal reinfected intradermally with 0.2 cc. of rabbit-virulent Type III pneumococcus blood broth culture at the indicated interval after first infection.

Rabbit	Character of original infection	Interval since first infection	Result
		<i>days</i>	
A	Single enzyme injection at 24 hrs., no further bacteriemia	12	D
B	Single enzyme injection at 24 hrs., no further bacteriemia	22	D
C	Single enzyme injection at 24 hrs., no further bacteriemia	27	D
D	Multiple injections of small amounts of enzyme; recurrent bacteriemia for 2 days	27	D
E	Multiple injections of small amounts of enzyme; recurrent bacteriemia for 5 days	27	S
F	Multiple injections of small amounts of enzyme; recurrent bacteriemia for 7 days	34	S
G	Multiple injections of small amounts of enzyme; recurrent bacteriemia for 9 days	34	S
H	Untreated animal which recovered after a long febrile course	22	S
I	Normal rabbit		D

S = survival of animal; localized lesion at point of inoculation; little or no elevation of temperature. High degree of immunity.

D = death of animal; lesion widespread; high temperatures; death at 3 to 5 days. No immunity.

by one of the writers in studying the immunity in animals after recovery following serum treatment (6).

## DISCUSSION

These experiments have shown that the enzyme which decomposes the capsular polysaccharide of Type III Pneumococcus has a marked

curative action in the disease brought about by infecting rabbits intradermally with a highly virulent strain of this organism. Following the injection of the enzyme in suitable amounts into the infected animal the blood stream becomes free of bacteria, the focal area of infection becomes sterilized, and the disease process ceases. A mortality rate of 5 per cent in treated cases is in sharp contrast to a mortality rate of 95 per cent in untreated rabbits.

The efficacy of the enzyme in infected rabbits might possibly have been predicted from its protective and curative action in mice. However, the infection in rabbits following intradermal injection of pneumococci is essentially different from the infection which occurs in mice following intraperitoneal injection. In the latter case a generalized infection rapidly occurs. In rabbits, on the other hand, the intradermal injection of pneumococci is followed by a disease which is primarily localized in the skin, and under these conditions the infecting organisms are less accessible to the action of the intravenously administered enzyme.

The present study has not dealt with the mechanism of the action of the enzyme, but in view of the findings of Avery and Dubos with mice, it seems probable that the enzyme brings about the rapid decomposition of the specific capsular polysaccharide, whether it be in the capsules of the bacteria or circulating free in the body fluids. The bacteria freed of their capsules may then be readily taken up and destroyed by the phagocytic cells of the infected animal.

#### SUMMARY

The action of the enzyme which specifically decomposes the capsular polysaccharide of Type III *Pneumococcus* has been tested in Type III pneumococcus dermal infections in rabbits. When injected in sufficient amounts, this enzyme is capable of bringing about a favorable and early termination of the experimental disease which ordinarily is fatal in nearly all instances.

The results of the present study yield further evidence that the capsular substance is of great importance in pneumococcus infection, since, in so far as known, the only action of which the specific enzyme is capable is that of decomposing the capsular polysaccharide.

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## CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

### VI. THE SYNTHESIS OF *p*-AMINOPHENOL $\alpha$ -GLUCOSIDE AND ITS COUPLING WITH PROTEIN

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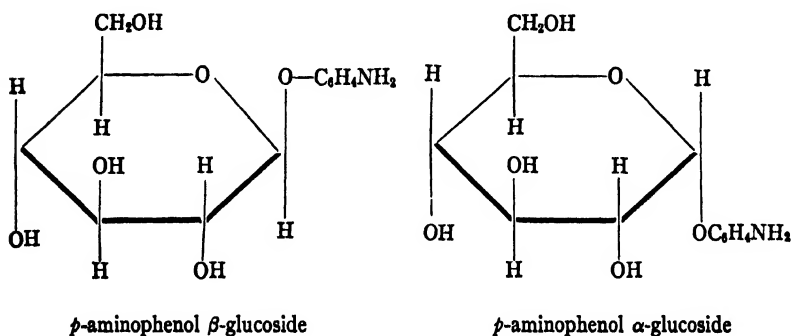
The preparation of synthetic carbohydrate-protein antigens has been described in previous publications from this laboratory (1). It will be recalled that simple hexose derivatives (aminophenol glucosides) have been attached to proteins by means of the diazo reaction. It was shown (2, 3) that when two chemically different carbohydrate derivatives are bound to the same protein, the newly formed substances exhibit a distinct immunological specificity and that this newly acquired specificity is determined by the chemical constitution of the carbohydrate radical attached to the protein molecule. Thus, simple differences in the molecular configuration of the two isomers, glucose and galactose, suffice to orientate specificity when the corresponding beta-aminophenol glucosides of these two sugars are coupled to the same or unrelated proteins.

The opportunity for differences in intermolecular chemical unions appears to be very great in the case of complex carbohydrates, such as the type-specific capsular polysaccharides of bacteria, which are built up of hexose and uronic acid molecules (4). As a result one might not only anticipate, but indeed one actually finds wide differences in immunological specificity among a group of closely related substances. We know but little of the intimate make-up of complex bacterial polysaccharides; still less do we know concerning the factors which orient their specificity. Although intermolecular stereochemical relationships, such as the position of linkage of one hexose molecule to another, exert an influence in orienting immune response, certain

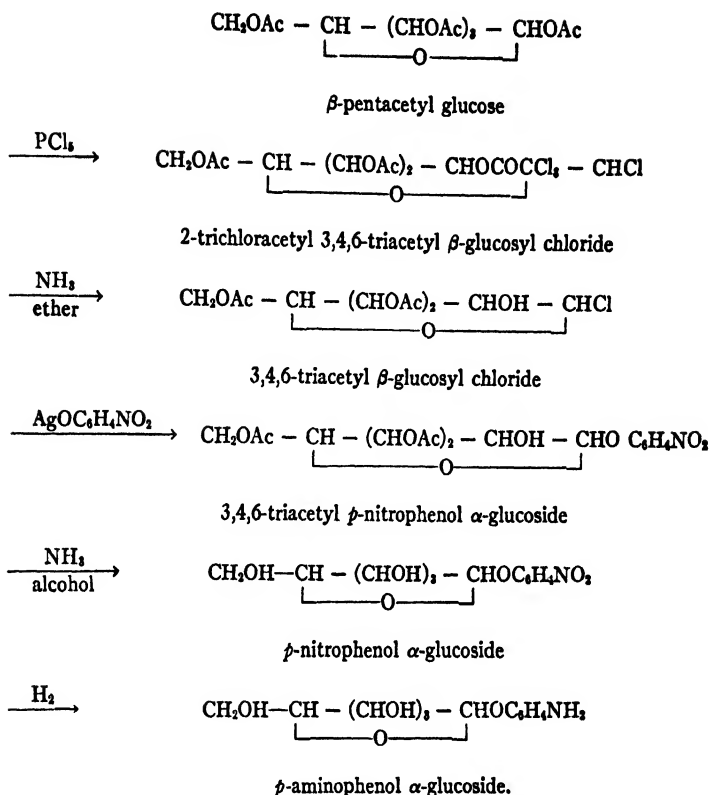
other factors (intramolecular) may assist in determining specificity; factors such as the presence of highly polar groups, stereochemical differences, etc.

The question of whether the type of hexose linkage (*i.e.* an alpha- or beta-glucosidic linkage) could exert an orienting influence on antigenic specificity appears both of interest and of importance. It is a problem approachable from the synthetic view point, and one which could be answered either by studying the immune response elicited by two disaccharides which differ from one another in the type of glucosidic linkage or, more simply, by studying the immunological specificity of two isomeric glucosides of the same hexose attached to the same protein.

We have undertaken the preparation of the alpha-*p*-aminophenol glucoside of glucose, and have coupled it to the globulin of horse serum. Its isomer, the beta-glucoside, has likewise been prepared (1). Thus two synthetic glucoproteins have been synthesized which differ from one another only in the alpha and beta linkage of the glucoside to the protein molecule. These differences in chemical constitution of the two glucosides can best be understood by the graphic formulae:



In the case of the alpha-glucoside, the aglucon (the non-sugar component) lies in a *cis* position in reference to the hydroxyl group in the second carbon atom of the hexose, whereas the aglucon of the beta-glucoside lies in the *trans* position. The method of preparation of the alpha-glucoside was accomplished by the following series of reactions:



## EXPERIMENTAL

(1) *β-Pentacetyl Glucose*.—This compound was prepared in the usual manner by the acetylation of glucose with acetic anhydride and sodium acetate.

(2) *2-Trichloracetyl 3, 4, 6-Triacetyl β-Glucosyl Chloride*.—This product was prepared from *β*-pentacetyl glucose by the action of phosphorus pentachloride according to the directions of Brigl (5). From 200 gm. of acetyl glucose 80 gm. of the recrystallized end-product were obtained, melting at 140°.

(3) *3, 4, 6-Triacetyl β-Glucosyl Chloride*.—The above compound, when treated at 0° with anhydrous ether saturated with dry ammonia gas (5), gave almost quantitative yields of the desired product. Recrystallization from anhydrous ethyl acetate yielded a product which melted at 154°.

(4) *Silver p-Nitrophenolate*.—This compound was prepared as previously described (1).

(5) *3, 4, 6-Triacetyl p-Nitrophenol  $\alpha$ -Glucoside*.—50 gm. of 3, 4, 6-triacetyl  $\beta$ -glucosyl chloride were suspended in 500 cc. of chloroform dried over phosphorus pentoxide. A large number of dry glass beads were placed in the bottle, and to the mixture were added, in three separate portions and at intervals of 10 minutes, 41.5 gm. (1.1 mols) of silver *p*-nitrophenolate. The mixture was shaken mechanically until no more chlorine could be detected in the chloroform solution. The mixture was then filtered by suction, and the precipitate was washed with chloroform. The combined filtrates were now concentrated to a syrup in vacuum. The residue, of dark brown color, was dissolved in 250 cc. of alcohol, and again evaporated to a syrup. After a second solution and evaporation the syrup was dissolved in alcohol and was placed overnight in the ice box. The glucoside crystallized out, it was filtered in the cold and washed with small portions of cold alcohol. 13 gm. of crude glucoside were recovered. The compound was twice recrystallized from alcohol, yielding about 11 gm. of a pure product melting at 148–149°C.

$$[\alpha]_D^{20} = \frac{+ 3.89 \times 100}{2 \times 2.467} = + 78.9^\circ \text{ (in methyl alcohol).}$$

Analysis: 4.659 mg. substance: 8.635 mg. CO<sub>2</sub> and 2.105 mg. H<sub>2</sub>O.

C<sub>18</sub>H<sub>21</sub>O<sub>11</sub>N. Calculated: C 50.58 per cent, H 4.95 per cent.

Found: C 50.54 per cent, H 5.05 per cent.

(6) *p-Nitrophenol Alpha-Glucoside*.—The triacetyl glucoside prepared as described above was dried at 60° in a vacuum oven. 20 gm. of the glucoside were dissolved in 75 cc. of anhydrous methyl alcohol and the solution added to 75 cc. of anhydrous methyl alcohol previously saturated with dry ammonia gas at 0°C. The mixture was placed in the ice box and allowed to stand for 15 hours. At the end of this time a large crystalline mass separated from the solution. Two distinct crystalline forms could be distinguished under the microscope, one form crystallized in needles, the other in prisms. The mixture was now warmed and the crystals redissolved. The solution was concentrated to dryness *in vacuo*, and was dissolved in 150 cc. of 95 per cent redistilled ethyl alcohol. The solution was again concentrated to dryness *in vacuo*. The crystalline mass was now dissolved in 175 cc. of 95 per cent ethyl alcohol, decolorized with a little norit and filtered. After standing overnight at room temperature (22–25°), the pure form of prismatic crystals was separated by filtration. 4.7 gm. were recovered. This compound, unrecrystallized, sintered at 205° and melted at 210–212°. After recrystallization from 60 cc. of ethyl alcohol glistening pale yellow crystals of the *p*-nitrophenol  $\alpha$ -glucoside of glucose were recovered which melted at 216–217° sintering slightly at first at 210°. The compound had an optical rotation of

$$[\alpha]_D^{20} = \frac{4.98 \times 100}{2 \times 1.093} = + 227.9^\circ \text{ (in methyl alcohol).}$$

Analysis: 4.692 mg. substance: 8.205 mg. CO<sub>2</sub> and 2.040 mg. H<sub>2</sub>O.

C<sub>12</sub>H<sub>16</sub>O<sub>8</sub>N. Calculated: C 47.83 per cent, H 5.02 per cent.

Found: C 47.68 per cent, H 4.86 per cent.

The original mother liquor from which the  $\alpha$ -glucoside was recovered, deposited, on cooling, a mixture of two crystalline forms. When the ethyl alcohol was removed by distillation and the substances subsequently dissolved in 150 cc. of methyl alcohol, and allowed to stand at room temperature, only the needle forms crystallized. This compound was filtered, and dried—8 gm. were recovered. When recrystallized from methyl alcohol glistening white needles melting at 164–165°C. were recovered. The compound had a specific optical rotation of  $-79.2^\circ$  in methyl alcohol, and was identified as *p*-nitrophenol  $\beta$ -glucoside after taking a mixed melting point with an authentic sample.

It was stated above that triacetyl  $\alpha$ -nitrophenol glucoside yielded a mixture of  $\alpha$ - and  $\beta$ -nitrophenol glucosides when the former was treated with anhydrous ammonia in methyl alcohol. This interesting phenomenon may possibly be ascribed to a mutarotation of the carbon atom bearing the aglucon group during the process of hydrolysis.<sup>1</sup> As substantiating evidence for this conception, it was observed that a sharp fall in the optical rotation of  $\alpha$ -nitrophenol glucoside occurred when an aqueous solution was permitted to stand in the presence of 0.5 normal ammonia at room temperature; a similar, though much less rapid drop in rotation occurred in 0.1 normal ammonia at 4°C. The substituted  $\alpha$ -nitrophenol glucoside appears, therefore, to be unstable in the presence of hydroxyl ions, and this instability is a function of the temperature and of the concentration of hydroxyl ions.

(7) *p*-Aminophenol  $\alpha$ -Glucoside.—2.0 gm. of *p*-nitrophenol  $\alpha$ -glucoside were dissolved in 100 cc. of warm ethyl alcohol. The substance was reduced catalytically with platinum oxide and hydrogen. From the alcoholic solution 1.4 gm. of the *p*-aminophenol  $\alpha$ -glucoside were isolated. This compound crystallized as a snow white product, readily soluble in water. It melted at 185–186°, and had a specific optical solution of

$$[\alpha]_D^{20} = \frac{5.35 \times 100}{2 \times 1.378} = +194.1^\circ \text{ (in methyl alcohol).}$$

A weighed sample, when titrated with standard nitrous acid, utilized the theoretical quantity.

<sup>1</sup> The authors wish to express their thanks to Dr. P. A. Levene for his interest and advice.



Analysis: 4.330 mg. substance: 8.389 mg.  $\text{CO}_2$  and 2.495 mg.  $\text{H}_2\text{O}$ .

$\text{C}_{12}\text{H}_{17}\text{O}_6\text{N}$ . Calculated: C 53.12 per cent, H 6.32 per cent.

Found: C 52.83 per cent, H 6.44 per cent.

(8) *Serum Globulin*.—Serum globulin was prepared by half saturating horse serum with ammonium sulfate, separating the globulins by centrifugation and again precipitating by half saturation. This process was repeated in all four times. The protein was finally dialysed and redissolved by the addition of solid sodium chloride. Thus pseudo- and euglobulin were separated from serum albumin. This solution of pseudo- and euglobulin was used for the preparation of protein-diazophenol  $\alpha$ -glucoside.

(9) *Preparation of Protein Diazophenol  $\alpha$ -Glucoside and  $\beta$ -Glucoside*.—The  $\alpha$ -glucoside was coupled to serum protein in the following manner.

120 mg. of  $\alpha$ -aminophenol glucoside were dissolved in 2.5 cc. of water. The solution was cooled to  $0^\circ\text{C}$ . and 1.0 cc. of normal  $\text{HCl}$  added. To this mixture was added 4.62 cc. of  $\text{N}/10$  sodium nitrite solution. After standing for 5 minutes at  $0^\circ\text{C}$ . the mixture was poured into an ice-cold solution of serum globulin, 300 mg. dissolved in 12 cc. of  $\text{N}/10$  sodium carbonate. The mixture was now permitted to stand for 30 minutes at  $0^\circ$ . At the end of this time the solution was carefully acidified to the point of maximum precipitation with 10 per cent trichloroacetic acid. The highly colored precipitate of  $\alpha$ -glucoside-azoglobulin was recovered by centrifugation and the supernatant liquid discarded. The precipitate was now suspended in 5 cc. of cold salt solution, stirred, and brought into solution by the cautious addition of  $\text{N}/10$   $\text{NaOH}$ . The deeply colored solution of sugar-azoprotein was now diluted to 20 cc. with salt solution, and the azoprotein reprecipitated by the careful addition of a few drops of  $\text{N}/10$   $\text{HCl}$ . The protein was again centrifuged and the supernatant liquid discarded. The precipitate was resuspended in cold saline, dissolved as before, and the final neutral solution diluted to 16 cc. One cc. was removed for a nitrogen analysis, and the sugar-azoprotein finally diluted so that each cc. contained 10 mg. of protein. This solution was sterilized by filtration through a Berkefeld candle, and used for immunization experiments, as described in the following paper.

The preparation of the  $\beta$ -glucoside-protein was carried out exactly as described above, except that pure  $\beta$ -*p*-aminophenol glucoside was used to couple with protein.

#### SUMMARY

1. The synthesis of *p*-aminophenol  $\alpha$ -glucoside has been described. This glucoside can be coupled to any protein to yield a synthetic  $\alpha$ -glucoside-protein complex.

2. A synthetic  $\beta$ -glucoside-protein complex has also been prepared.

3. These synthetic sugar-protein complexes have been used as immunizing antigens in order to ascertain whether  $\alpha$ - and  $\beta$ -glucosidic

unions influence the specificity of the immune response in animals. The results of the immunological studies are presented in the following paper.

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## CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

### VII. IMMUNOLOGICAL SPECIFICITY OF ANTIGENS PREPARED BY COM- BINING $\alpha$ - AND $\beta$ -GLUCOSIDES OF GLUCOSE WITH PROTEINS

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The study of antigens prepared by chemically combining sugar derivatives with protein has revealed the fact that carbohydrates exert a determining influence on the immunological specificity of the compounds of which they form a part. In preceding papers evidence has been presented showing that mere differences in the structural configuration of a single carbon atom may determine the serological specificity of two sugar derivatives identical save in this one respect.

As previously pointed out, the *p*-aminophenol  $\beta$ -glucosides of glucose and galactose, differing from each other only in the interchange of the H and OH groups on a single carbon atom, exhibit an individual specificity irrespective of the protein to which they are attached (1, 2). Moreover, the specific rôle of the sugar radical in the reactive part of the antigen is revealed by the fact that the glucosides alone, unattached to protein, specifically inhibit the precipitin reaction between the corresponding antiserum and homologous antigen.

The inhibition of the antigen-antibody reaction by the intervention of the specific sugar hapten was found to occur both *in vitro* and *in vivo*. In guinea pigs passively sensitized with the precipitating serum of immune rabbits, the fatal anaphylactic shock which invariably follows the intravenous administration of the homologous sugar-protein was completely prevented by a single prophylactic injection of the specific glucoside alone. The unconjugated glucosides, although themselves not capable of inducing shock, specifically inhibited the anaphylactic reaction in sensitive animals when given immediately before the introduction of the shock-producing antigen (3). The specificity of the  $\beta$ -glucosides of glucose and galactose was demonstrated by the fact that the inhibition of the anaphylactic reaction, like the inhibition of the precipitin test *in vitro*, occurred only when the glucoside used was the same as that present in the specifically reacting antigen.

These earlier studies concerned themselves with the specificity of the  $\beta$ -glucosides of two different hexoses, glucose and galactose. The present paper deals with the specificity of two different glucosides of the same sugar, namely, the *p*-aminophenol  $\alpha$ - and  $\beta$ -glucosides of glucose.

The " $\alpha$  and  $\beta$  antigens" formed by the union of the diazonium derivatives of these glucosides with protein differ from each other only in the spatial relations of the terminal carbon atom in each sugar component. Since the ultimate composition of both antigens is chemically the same, any observable differences in their immunological specificity can be referable only to known differences in the molecular structure of each. The following experiments were carried out to determine to what extent two isomeric glucosides of the same sugar, in combination with a single protein, would influence the specificity of two antigens otherwise identical. The present paper deals with the question of the specificity of the precipitins present in the serum of rabbits immunized with  $\alpha$ - and  $\beta$ -gluco-globulin.

## EXPERIMENTAL

### *Methods*

The synthesis of the  $\alpha$ - and  $\beta$ -*p*-aminophenol glucosides of glucose and the method of coupling the diazonium derivatives to protein are given in detail in the preceding paper (4). The method of the intravenous immunization of rabbits and the technique of the precipitin and inhibition tests differ in no essential respect from those described in the earlier studies (2).

The *immunizing antigens* were prepared by combining each of the glucose derivatives to globulin derived from normal horse serum; on the other hand the *test antigens* used in the precipitin reactions, contained the respective glucosides bound to the proteins of chicken serum. The use of proteins of remote biological origin in the two sets of antigens excludes the possibility of common antiprotein precipitins masking the specificity of the carbohydrate reactions.

### *I. Anticarbhydrate Antibodies*

(1) *Specific Precipitin Reactions.*—The sera prepared by immunization of rabbits with  $\alpha$ - and  $\beta$ -gluco-globulin were first tested for the presence of precipitins specifically reactive with an antigen containing the same carbohydrate radical. The test antigens employed in the precipitin reactions were in each instance prepared by combining the

respective glucoside with a protein unrelated to that used in the immunizing antigen. Under these conditions, the results of the tests are not confused by the presence of a common protein and the specificity of the reactions are directly interpretable in terms of known differences in the chemical structure of sugar radical in the reactive part of each antigen.

TABLE I  
*Specific Precipitins in Sera of Rabbits Immunized with  $\alpha$ -Gluco-Globulin\**

Immune rabbit sera	Test antigen: $\alpha$ -gluco-chicken serum*			
	1:5,000	1:10,000	1:20,000	1:40,000
1	++++	+++	++	+±
2	++	++	+	±
3	+++	+++	++	+

++++ = Complete precipitation with compact disk-like precipitate.

\* In order to avoid the reactions of a common protein, the *immunizing antigen* was prepared by combining  $\alpha$ -glucoside with the globulin from horse serum, while the *test antigen* was similarly prepared by coupling the same glucoside to the proteins of chicken serum.

TABLE II  
*Specific Precipitins in the Sera of Rabbits Immunized with  $\beta$ -Gluco-Globulin*

Immune rabbit sera	Test antigen: $\beta$ -gluco-chicken serum*			
	1:5,000	1:10,000	1:20,000	1:40,000
1	+++	++	+±	+
2	++++	++++	+++	+++
3	++++	++++	+++	+++

++++ = Complete precipitation with compact disk formation.

\* Unrelated proteins in immunizing and test antigens (see footnote, Table I).

The precipitin reactions between the homologous test antigens and the antisera of rabbits immunized with  $\alpha$ - and  $\beta$ -gluco-globulin are given in Tables I and II.

The results of the precipitin tests illustrate the capacity of the immune sera to react with an antigen containing the homologous glucoside irrespective of the protein to which it is attached. They confirm the earlier observations concerning the orienting influence of the

sugar radical on the specificity of the protein with which it is combined.

(2) *Specific Inhibition Tests*.—The selective specificity of the antibodies reactive with the two isomeric derivatives of glucose is clearly demonstrated by the results of the inhibition tests given in Tables III and IV.

Analysis of the data presented in Table III shows that the  $\alpha$ -glucoside alone, when added in excess to the homologous antiserum specifically binds the precipitins and thus renders them unable to react subse-

TABLE III

*Specific Inhibition of Precipitins in  $\alpha$ -Glucoglobulin Antiserum by Homologous Glucoside*

$\alpha$ -gluco-globulin antiserum		Glucosides m/15		Salt solution to volume		Test antigen: $\alpha$ -gluco-chicken serum*			Result
		$\alpha$	$\beta$			1:2,000	1:5,000	1:10,000	
	cc.	cc.	cc.	cc.	2 hrs. at 37°C. No precipitation	cc.	cc.	cc.	
1	0.2	—	—	0.3		0.5	—	—	+++
2	0.2	—	—	0.3		—	0.5	—	+++
3	0.2	—	—	0.3		—	—	0.5	+++
4	0.2	0.3	—	—		0.5	—	—	0
5	0.2	0.3	—	—		—	0.5	—	0
6	0.2	0.3	—	—		—	—	0.5	0
7	0.2	—	0.3	—		0.5	—	—	++±
8	0.2	—	0.3	—		—	0.5	—	+++
9	0.2	—	0.3	—		—	—	0.5	+++

+++ = Disk-like precipitate

0 = No precipitation, showing complete inhibition.

\* See footnote, Table I.

quently with the test antigen containing the same sugar derivative. The specificity of this inhibition is shown by the fact that the addition of an equal concentration of the  $\beta$ -glucoside to  $\alpha$  immune serum has little or no appreciable effect on the precipitin reaction with the  $\alpha$  antigen. Similar relationships are shown to exist in the specific inhibition of the precipitins in  $\beta$  antiserum by the  $\beta$ -glucoside (Table IV).

The results of the inhibition tests reveal the specificity of the anti-carbohydrate reactions, since in the case of both  $\alpha$  and  $\beta$  antisera the precipitins for the homologous antigen are inhibited only by the corre-

sponding glucoside. Although the glucosides by themselves, unattached to protein, fail to produce precipitation in immune sera, each by binding the specific antibodies in its homologous antiserum prevents them from again reacting with a test antigen containing the same glucoside. In this sense the glucosides unattached to protein function as haptens, the chemo-specific groups of which unite with the corresponding antibodies without causing visible change in the reaction mixture. Marrack and Smith (5) using a direct method have

TABLE IV

*Specific Inhibition by Homologous Glucoside of Precipitins in  $\beta$ -Gluco-Globulin Antiserum*

$\beta$ -gluco-globulin antiserum		Glucosides $\mu/15$		Salt solution to volume		Test antigen: $\beta$ -gluco-chicken serum*			Result
		$\alpha$	$\beta$			1:2,000	1:5,000	1:10,000	
	cc.	cc.	cc.	cc.	2 hrs. at 37°C. No precipitation	cc.	cc.	cc.	
1	0.2	—	—	0.3		0.5	—	—	++++
2	0.2	—	—	0.3		—	0.5	—	++++
3	0.2	—	—	0.3		—	—	0.5	++++±
4	0.2	—	0.3	—		0.5	—	—	0
5	0.2	—	0.3	—		—	0.5	—	0
6	0.2	—	0.3	—		—	—	0.5	0
7	0.2	0.3	—	—		0.5	—	—	++++
8	0.2	0.3	—	—		—	0.5	—	++++
9	0.2	0.3	—	—		—	—	0.5	++++

++++ = Complete precipitation with disk-like precipitate.

0 = No precipitation, showing complete inhibition.

\* See footnote, Table I.

recently brought experimental proof of Landsteiner's original view that the union between hapten and antibody is a specific combination between the chemo-specific groups of the reacting agents. As Landsteiner has pointed out in the case of non-protein radicals which function as haptens, the union of these glucosides with antibody does not lead to precipitation unless the reacting groups are attached to protein.

The preceding experiments have dealt with the homologous immune reactions of two antigens of known chemical constitution which differ one from the other only in the  $\alpha$  and  $\beta$  type of glucosidic union. The results show that mere differences in the spatial arrangement of the



groups on the terminal carbon atom in these two derivatives of glucose suffice to confer on each antigen a marked degree of differential specificity. For, as shown by the specific inhibition reactions, it is possible to differentiate selectively between the two isomeric glucosides of the same sugar by the use of immune sera.

Despite the reflection in serological specificity of differences in the stereochemical relationships of the first carbon atom bearing the non-sugar constituent of each glucoside, it must be borne in mind that in both the  $\alpha$  and  $\beta$  derivatives the spatial arrangement of the polar groups on the remaining five carbon atoms is identical. Quite different, however, are the structural relations existing in the case of the  $\beta$ -glucosides of glucose and galactose, the immunological specificity of which has previously been shown to be absolute (2). In these latter glucosides the stereochemical arrangement of the groupings on the terminal carbon atom are both in the  $\beta$  position, but the symmetry of the polar groups on the remaining five carbon atoms is not identical, for on the fourth carbon atom of galactose the H and OH groups are rotated through an angle of  $180^\circ$ . This difference in molecular configuration, with its consequent change in the spatial relationship of the polar groups, is the important if not the sole factor determining the individual specificity of the  $\beta$ -glucosides of glucose and galactose. On the basis of these facts, one might anticipate an absolute specificity in the case of the similar glucosides of two different hexoses, but an overlapping specificity in isomeric glucosides of the same hexose.

(3) *Cross-Precipitin Reactions*.—It is therefore not surprising to find that this partial similarity in the chemical structure of the  $\alpha$  and  $\beta$  derivatives of glucose is reflected in the serological relationships of both substances. The results of the cross-precipitin tests presented in Table V show that antibodies present in  $\alpha$  and  $\beta$  antisera cross-react in each instance with the heterologous test antigen.

The cross-precipitin reactions are sharply defined and are quantitatively only slightly less than the reactions between each antiserum and its homologous antigen.

Interesting relationships in the overlapping specificity of both sugar derivatives are brought out in the reciprocal inhibition tests, the results of which are given in Table VI.

Analysis of the data presented in Table VI shows that the addition of  $\alpha$ -glucoside to  $\alpha$  antiserum inhibits the precipitins from reacting

with both  $\alpha$  and  $\beta$  test antigens. However, the addition of an equal concentration of  $\beta$ -glucoside to  $\alpha$  antiserum inhibits the precipitin

TABLE V

*Cross-Precipitin Reactions of  $\alpha$ - and  $\beta$ -Gluco-Globulin Antisera with Heterologous Antigens*

Immune sera	Test antigens*					
	$\alpha$ -gluco-chicken serum			$\beta$ -gluco-chicken serum		
	1:5,000	1:10,000	1:20,000	1:5,000	1:10,000	1:20,000
$\alpha$ -gluco-globulin.....	++++	+++	++	++±	++	+
$\beta$ -gluco-globulin.....	++	++	+	++++	++++	++++

++++ = Complete precipitation with compact disk-like precipitate.

\* See footnote, Table I.

TABLE VI

*Inhibition of Cross-Precipitin Reactions by Homologous and Heterologous Glucoside*

Gluco-globulin antisera	Glucosides m/15		Salt solution to volume		Test antigen* 1:10,000		Results
	$\alpha$	$\beta$			$\alpha$	$\beta$	
$\alpha$ .	$\alpha$ .	$\alpha$ .	$\alpha$ .	2 hrs. at 37°C.	$\alpha$ .	$\alpha$ .	
$\alpha$ 0.2	—	—	0.3		0.5	—	+++
$\alpha$ 0.2	0.3	—	—		0.5	—	0
$\alpha$ 0.2	—	0.3	—		0.5	—	++±
$\alpha$ 0.2	—	—	0.3		—	0.5	++
$\alpha$ 0.2	0.3	—	—		—	0.5	0
$\alpha$ 0.2	—	0.3	—		—	0.5	0
$\beta$ 0.2	—	—	0.3	2 hrs. at 37°C.	—	0.5	++++
$\beta$ 0.2	—	0.3	—		—	0.5	0
$\beta$ 0.2	0.3	—	—		—	0.5	++++±
$\beta$ 0.2	—	—	0.3		0.5	—	++
$\beta$ 0.2	—	0.3	—		0.5	—	0
$\beta$ 0.2	0.3	—	—		0.5	—	0

Plus signs indicate positive reaction with gradation in amount of precipitate.

0 = no precipitation, showing complete inhibition.

— = reagent not used.

\* See footnote, Table I.

reaction with the  $\beta$  test antigen without appreciably diminishing its capacity to precipitate the  $\alpha$  antigen. Similar relationships hold in

the case of the  $\beta$  antiserum with respect to the inhibition of precipitins by the homologous and heterologous glucosides.

It is evident from these results, that the addition of the homologous glucoside to its antiserum completely inhibits the precipitins for both the homologous and heterologous test antigens. On the other hand, the addition of the heterologous glucoside to immune serum inhibits only the precipitins for the heterologous test antigen and has but slight effect upon the antibodies reactive with the homologous antigen. The results of the cross-inhibition tests with the  $\alpha$ - and  $\beta$ -glucosides show that the reaction of an immune serum with its homologous antigen is specifically inhibited only by the homologous glucoside; while the cross-reaction between this serum and the heterologous antigen is

TABLE VII

*Precipitins for Globulin in Sera of Rabbits Immunized with  $\alpha$ - and  $\beta$ -Gluco-Globulin\**

Immune sera	Globulin from horse serum			
	1:5,000	1:10,000	1:20,000	1:40,000
$\alpha$ -gluco-globulin.....	+	$+\pm$	++	++
$\beta$ -gluco-globulin.....	+	++	++	+++

+++ = flocculent precipitate, not compact.

\* Both immunizing antigens were prepared by combining each glucoside with globulin from horse serum.

completely inhibited by either glucoside. This lack of reciprocal inhibition of the precipitins in  $\alpha$  and  $\beta$  antisera may be interpreted as further evidence of the lack of the immunological identity of the two isomeric glucosides.

## II. Antiprotein Antibodies

As previously described the immunizing antigens were prepared by combining the  $\alpha$  and  $\beta$  derivatives of glucose with globulin obtained from normal horse serum. The sugar-protein antigens were standardized on the basis of their nitrogen content so as to contain 5 mg. of protein per cc. Each rabbit received during the course of immunization a total of approximately 160 mg. of protein antigen.

In addition to the antibodies reactive with the specific glucosides, there are also present in both immune sera precipitins for the globulin used in preparing the immunizing antigens (Table VII).

The precipitating antibodies for horse serum globulin may arise in response to the presence in both antigens of free protein unbound by the diazotized glucosides. However, it is also possible that even in the absence of free protein, the sugar-protein antigen as a whole may stimulate the formation of two qualitatively different antibodies, each specifically related to the corresponding component of the antigenic complex. This concept of the dual antigenicity of a single complex antigen involves the assumption that the coupling of the glucoside to the protein has not masked the groups essential to the specificity of the protein, and that while the sugar radical through conjugation acquires specific antigenicity, the protein molecule retains, in part at least, its original antigenic properties.

#### DISCUSSION

Landsteiner and Lampl (6), working with ortho, meta, and para substituted aromatic compounds, have pointed out the significance of the spatial arrangement of the groupings upon which the specificity of these compounds depends. In more recent studies on the serological differentiation of steric isomers of *p*-aminobenzoylamino acetic acid and tartaric acid, Landsteiner and van der Scheer (7) have further emphasized the dependence of immunological specificity upon chemical structure.

The results of the present study add further evidence in support of the view that the immunological specificity of carbohydrates is determined by their chemical constitution. Differences in the specific behavior of the  $\alpha$ - and  $\beta$ -glucosides of glucose may be accounted for by known differences in the spatial position of the groups on the first carbon atom of each glucoside. These structural changes are so sharply reflected in serological specificity that it is possible by means of immune sera to differentiate selectively between the two isomeric glucosides of the same sugar. However, granting the difference in the chemical structure of the two glucosides it becomes necessary to account for their overlapping specificity. The chemical basis for this immunological crossing may lie in the fact that the spatial arrangement of the polar groups on the remaining five carbon atoms is identical in both glucosides. This partial similarity of molecular grouping might then account for the degree of immunological likeness exhibited

by the two substances. Although in a portion of the molecule of both glucosides the structural relationship is identical, nevertheless they behave chemically as separate entities and possess serologically a separate and distinct specificity. If complete reciprocal inhibition of precipitins is accepted as the criterion of serological identity then the failure of both glucosides to exhibit this capacity may be taken as further evidence of differences in the immunological specificity of each.

The lack of reciprocal absorption of agglutinins by two organisms mutually agglutinable in the immune serum of each is generally conceded to indicate a lack of immunological identity. Relationships of this order are known to exist between *Pneumococcus* Type II and *Friedländer bacillus* Type B. In both instances the reactive substance has been identified chemically as the specific polysaccharide peculiar to the capsule of each organism (8). While the structural constitution of these complex sugars is not as yet fully known, considerable knowledge has been gained concerning their chemical properties. For example, it is known that the *Pneumococcus* Type II polysaccharide is built up of glucose units and that chemically it bears a close resemblance to the polysaccharide recovered from the Type B *Friedländer bacillus*. However, the two substances are not chemically identical, although the similarity between them is sufficient to result in a certain likeness in immunological specificity (9). In the absence of precise knowledge of the structural relations of the two polysaccharides, it seems reasonable to assume that both contain in a portion of the complex molecule the same or a closely similar configuration of atoms. This similarity of molecular grouping might then account for the immunological similarity of the two substances.

Considerable evidence for this point of view is found in the results of the present study concerning the specificity of the  $\alpha$ - and  $\beta$ -glucosides of glucose. A comparison of the serological relationships between the two isomeric derivatives of glucose and the capsular polysaccharides of the two organisms in question is presented in Table VIII.

Analogous relationships extending even to the cross-immunity reactions and the lack of reciprocal absorption and inhibition of antibodies are evident in both the groups of simple and complex carbohydrates. While the comparison is instructive it is not necessarily valid in the case of the capsular polysaccharides, for the final interpretation of

these relationships must await further knowledge of the structural relations of the more complex bacterial sugars.

TABLE VIII

*Comparison of the Serological Relationship between Isomeric Derivatives of Glucose and the Capsular Polysaccharides of Two Unrelated Species of Bacteria*

$\alpha$ -gluco-globulin antiserum			Antipneumococcus serum Type II		
By addition of	Precipitins are		Absorbed with	Agglutinins are	
	inhibited for	not inhibited for		removed for	not removed for
$\alpha$ -glucoside	$\alpha$ and $\beta$ test antigens		Pneumococcus Type II	Pneumococcus Type II <i>B. friedlaenderi</i> Type B	
$\beta$ -glucoside	$\beta$ test antigen	$\alpha$ test antigen	<i>B. friedlaenderi</i> Type B	<i>B. friedlaenderi</i> Type B	Pneumococcus Type II
$\beta$ -gluco-globulin antiserum			Anti- <i>friedlaenderi</i> serum Type B		
By addition of	Precipitins are		Absorbed with	Agglutinins are	
	inhibited for	not inhibited for		removed for	not removed for
$\beta$ -glucoside	$\alpha$ and $\beta$ test antigens		<i>B. friedlaenderi</i> Type B	Pneumococcus Type II <i>B. friedlaenderi</i> Type B	
$\alpha$ -glucoside	$\alpha$ test antigen	$\beta$ test antigen	Pneumococcus Type II	Pneumococcus Type II	<i>B. friedlaenderi</i> Type B

The test antigens in all instances were prepared by combining the respective glucoside with a protein biologically unrelated to that in the immunizing antigen; see footnote, Table I.

Enders (10) has recently suggested that cross-relationships between Type II *Pneumococcus* and Type B *Friedländer* bacillus may be

due to the presence in pneumococci of a type-specific agglutinin unrelated to the specific carbohydrate.

In the case of the synthetic antigens containing the  $\alpha$  and  $\beta$  compounds of glucose alone, the evidence indicates that the immunological relationships of the reactive glucosides are determined by known variations in their chemical constitution and are independent of the protein to which they are attached. In view of these findings it seems not unlikely that in the case of the polysaccharides, because of their more complicated structure and the greater possibility for variation in molecular configuration, there may be found many examples of a similar overlapping specificity among carbohydrates of unrelated origin.

#### SUMMARY

The chemical and immunological properties of the *p*-aminophenol  $\alpha$ - and  $\beta$ -glucosides of glucose are described and correlated. The results are discussed with reference to their possible bearing on the chemo-immunological nature of the specific polysaccharides of bacterial origin.

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## REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

### VII. THE RELATION OF HYPERSENSITIVENESS TO LESIONS IN THE LUNGS OF RABBITS INFECTED WITH PNEUMOCOCCI

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Certain writers have drawn attention to the effect of active immunization or sensitization (1-6) on the occurrence or character of lesions in the lungs of animals artificially infected with pneumococci. On the basis of these observations some investigators have attempted to explain the pathogenesis of pneumonia in man. In a recent paper, Stuppy, Cannon, and Falk (7) report that after daily insufflation of heat-killed pneumococci, rabbits acquire a type-specific immunity and the reaction of the lung following intratracheal injections of cultures of corresponding types consists of proliferation and exudation with the macrophage the predominant cell. Polymorphonuclear and eosinophilic cells were also present. A similar though less extensive reaction was induced by the intratracheal injection of cultures of heterologous types. Sharp and Blake (8) have shown that the intratracheal injection of an autolysate of *Pneumococcus* induces no reaction in the lung of normal rabbits but in rabbits previously inoculated with bacteria or autolysate similar injections stimulate inflammatory changes in the lung which are parallel in severity with the skin reactivity of these rabbits to the same material.

In the previous papers of this series, studies have been reported which indicate that rabbits undergo different forms of sensitization to pneumococci and their constituents depending upon the route and mode of injection. It has been shown that when rabbits receive repeated intracutaneous or intravenous injections of pneumococci or solutions of their protein constituents (9), they acquire a heightened sensitivity, so that when later intracutaneous injections of nucleoprotein are made, a local reaction occurs which is similar to that seen in the Arthus phenomenon. This skin sensitivity does not seem to be associated with a state of increased resistance to infection.

In rabbits that have been repeatedly injected intracutaneously with suspensions of dead pneumococci, there occurs in addition to the heightened skin reactivity, a condition of heightened eye sensitivity (10) which is manifested by an inflammatory reaction when there is instilled in the eyes a solution of pneumococcus nucleoprotein or a solution of pneumococci from which the acid- and heat-coagulable proteins have been removed. It was found that the eye sensitivity does not occur



when the previous injections are made by routes other than the skin, and it develops only after intact bacteria are injected. The heightened eye reactivity apparently occurs only in animals that are resistant to infection.

In the light of these observations, it has seemed important to investigate further the possibility of sensitization of the tissues of the lung to pneumococci and their products and to study the relationship of this phenomenon to actual infection with pneumococci.

The first experiments bearing on the problem consisted in determining whether rabbits which had been sensitized previously by repeated injections of a foreign protein would show an increased reaction in the lung following the intratracheal injection of the same protein.

Opie (11) has called attention to the occurrence of hypersensitiveness in tissues other than the skin, and has described the occurrence of localized consolidation in the lung following the injection of 0.2 cc. of horse serum through the thoracic wall in a rabbit which had previously received repeated injections of serum. Accordingly, rabbits were injected repeatedly either intracutaneously or intravenously with crystalline egg albumin as previously described. 3 weeks after the last injection the skin reacted severely to injection of the homologous protein but no sensitivity of the eye could be demonstrated. After observations had been made on eye and skin reactivity, solutions of egg albumin containing 3 to 10 mg. of the protein were injected intratracheally and the animals were killed 24 hours later by the intravenous injection of air. Upon histological examination, the lungs showed different degrees of reaction. In some instances definite pneumonic areas were observed. The alveolar walls were infiltrated and the alveoli were filled with an exudate containing chiefly mononuclear and polymorphonuclear cells. In other instances no definite changes were seen.

It is obvious from this experiment that certain rabbits which have received repeated injections of a foreign protein are so sensitized that an acute reaction occurs in the lung when the protein solution is reinjected intratracheally.

It was now important to repeat this experiment, employing however, instead of egg albumin, the nucleoprotein derived from pneumococci. Using the same technique, similar results were obtained.

Another series of animals were now sensitized by repeated intravenous or intracutaneous injections of whole heat-killed pneumococci, instead of merely the bacterial protein. On subsequent intratracheal injections of the bacterial protein, these animals reacted just as did those in the previous experiments.

Experiments were then undertaken to determine whether this state of hypersensitiveness, the presence of which could be demonstrated by the intratracheal injection of protein, could be passively transferred from one animal to another by the injection into a normal animal of the serum from a sensitized one.

Each of nine normal rabbits was injected intravenously with 14 to 30 cc. of serum derived from a single animal which gave typical skin reactions to the respective antigens. Three of the rabbits received serum from rabbits injected with egg albumin, three received serum from rabbits injected with pneumococcus protein, and three received serum from rabbits injected with heat-killed pneumococci. On the day following the transfer of serum, the recipient animals were found to possess precipitins in their sera and exhibited reactions in the skin following injections of the specific antigen. Eye sensitivity was tested but in no instance was a positive reaction obtained. The introduction of the respective protein intratracheally induced in the lung a lesion comparable to that observed in the actively sensitized animals. In three of the animals the reaction in the lung was of the more severe variety while in the others the reaction was mild and diffuse or even doubtful.

An attempt was made to determine whether the lung reaction could also be elicited by the intratracheal injection of a solution of pneumococci from which the acid- and heat-coagulable proteins had been removed. The reactions produced in normal rabbits by this material were so severe, however, that no conclusions could be drawn.

These experiments indicate that in rabbits a state of sensitization to foreign proteins, including pneumococcus nucleoprotein, may be induced so that upon the intratracheal injection of this protein inflammatory reactions occur in the lung parenchyma. That this sensitivity may be passively transferred, and that it follows both intravenous and intracutaneous inoculations indicate that it is analogous to the heightened skin reactivity in rabbits (12), and that it differs from the eye sensitivity previously described.

Experiments were then undertaken to determine, if possible, the effect of such a state of hypersensitivity on actual infection with pneumococci.

Preliminary studies in normal animals, employing the same technique as was used later in the sensitized ones, showed that following the intravenous injection of small quantities ( $10^{-5}$  to  $10^{-7}$  cc.) of broth cultures of *Pneumococcus*, the animals

died usually within 24 to 48 hours, more rarely 72 hours, with a marked septicaemia. The lungs showed varying degrees of involvement. Frequently there were found inflammatory reactions, alveolar exudation, and in some instances, hemorrhages. Rabbits which had previously received repeated intravenous or intracutaneous injections of heat-killed pneumococci were not similarly infected. As these animals were more or less resistant to the infection they were killed in from 1 to 5 days following inoculation by the intravenous injection of air. The majority of these animals showed little or no change in the lung. In those dying 4 to 5 days following the infection, however, quite marked changes were frequently observed. In the lungs of some of these rabbits there was a distinct pneumonic process consisting of an enormous increase of mononuclear and polymorphonuclear leucocytes. These cells filled the alveoli distending the walls to form an area of consolidation. Occasionally bronchioles were found containing numbers of necrotic cells. Usually, the cellular reaction was principally mononuclear. In a small number of animals dying of the infection, extensive empyema and massive pericarditis was found.

Even in these partially resistant animals the lesions differed from those seen in previously untreated rabbits merely in severity and in the occasional occurrence of empyema and pericarditis.

As previously stated, only certain of the animals showed lesions in the lungs. It was established that it could not be predicted which of the animals would show pulmonary lesions and which would not. Certain of the animals were prepared by repeated intracutaneous injections of whole pneumococci, and in the majority of instances, these were eye sensitive. There was no difference in the character or frequency of the pulmonary lesions in the animals which were eye sensitive and in those which were not.

Finally an attempt was made to determine whether when infection was induced in rabbits already undergoing a pulmonary protein reaction, the lesions in the lung would be markedly different from those occurring in the similarly infected normal animal. For this purpose rabbits were previously injected either intracutaneously or intravenously with heat-killed pneumococci until their sera showed a high titre of antiprotein precipitins. About 3 weeks after the last injection of bacteria, pneumococcus protein was introduced intratracheally and 24 hours later virulent cultures of the organism were injected intravenously. A study of the lung reaction to infection in these rabbits showed no essential difference from that occurring in sensitized rabbits which had not received nucleoprotein intratracheally on the preceding day.

## DISCUSSION

The studies reported in the present communication were undertaken to determine whether a preexisting state of hypersensitiveness to *Pneumococcus* or its products influences the course and character of pneumococcus infection in the lungs of rabbits. The study was prefaced by observations on the reaction of the lung to soluble proteins in sensitive animals. It was found that the intratracheal injection of native or pneumococcus proteins may induce inflammatory reactions of the lung in animals previously injected with the respective antigen. Moreover, similar reactions follow the intratracheal injection of pneumococcus protein in rabbits that have previously received inoculations of heat-killed pneumococci. That the reaction induced by intratracheal injections of protein is related to the presence of circulating precipitins was shown by the occurrence of the lung reaction, following the transfer of serum from a reactive to a normal rabbit.

A study of the response of the lung to infection in animals made resistant or sensitive by the repeated intravenous or intracutaneous injections of pneumococci shows no appreciable difference attributable to the route of preliminary administration of the bacteria. Infection was accomplished by the intravenous injection of many lethal doses of virulent organisms, and the reaction in the lung occurred neither frequently nor regularly. As far as could be determined by this method of infection the response of the lung is not different in normal animals and in rabbits which have previously received intravenous or intracutaneous injections of pneumococci. The presence of eye or skin hypersensitiveness appears to have no influence on the course or character of the induced infection.

In order to test the possibility that an antigen-antibody reaction in the lung might increase the severity of the infection, pneumococcus protein was first injected intratracheally in sensitized rabbits and on the following day the rabbits were infected by the intravenous injection of pneumococci. The presence of an intrapulmonary reaction at the time of infection did not have any apparent effect on the course of the disease.

In brief, it can be stated that the present study fails to show that hypersensitiveness to *Pneumococcus* or its products influences the occurrence or character of the lesions in the lungs in artificial pneumococcus infections.

## SUMMARY AND CONCLUSION

1. The intratracheal injection of egg albumin or pneumococcus protein induces an inflammatory reaction in the lungs of rabbits previously inoculated with the respective antigen.

2. A similar reaction occurs following intratracheal injection of pneumococcus protein into the lungs of rabbits previously inoculated with heat-killed suspensions of the bacteria.

3. This reaction appears to be related to the presence of circulating antibody and to have the nature of the Arthus reaction.

4. A study of the reaction of the lung of rabbits to infection caused by intravenous injections of *Pneumococcus* reveals that (a) reactions occur irregularly in the lung; (b) in the lungs in which reactions do occur, the histological changes are not different in normal rabbits and in rabbits made resistant by previous intravenous or intracutaneous injections of pneumococci.

5. Intratracheal injection of pneumococcus protein followed by intravenous injection of virulent pneumococci on the next day does not alter the course and character of the infection in resistant rabbits.

6. The experiments reported in this paper bring no evidences to support the view that the lesions in the lungs of rabbits following the intravenous injection of pneumococci are modified by any previous state of sensitivity.

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## DAS GEWEBSBILD DES FIEBERHAFTEN RHEUMATISMUS

### V. MITTEILUNG

#### UNTERSUCHUNGEN DES RHEUMATISCHEN FRÜHINFILTRATS AUF STREPTOKOKKEN

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Im Laufe der letzten vier Jahrzehnte ist eine große Zahl verschiedener Mikroorganismen als Erreger des fieberhaften Rheumatismus angesprochen worden. Die meisten von ihnen hält man jetzt entweder für Verunreinigungen oder sekundäre Infektionen; nur die Streptokokken, denen zuerst von *Singer*<sup>1</sup> für den Gelenkrheumatismus die Rolle des Erregers zugesprochen wurde, werden auch heute noch von vielen Forschern dafür gehalten. Einige sind der Ansicht, daß bestimmte Typen von Streptokokken als spezifische Erreger in Frage kommen, während andere die Grundlage des Gelenkrheumatismus in einem Zustand der Überempfindlichkeit gegenüber verschiedenen Streptokokken sehen, und vielleicht auch gegenüber anderen Mikroorganismen. Die Aufmerksamkeit an der Streptokokkentheorie wurde neuerdings wieder lebendig durch die Arbeiten von *Small*<sup>2</sup>, *Birkhaug*<sup>3</sup> und von *Cecil, Nicholls* und *Stainsby*<sup>4</sup>; die Theorie der Streptokokkenüberempfindlichkeit dagegen wurde von *Herry*<sup>5</sup>, *Faber*<sup>6</sup>, *Swift* und seinen Mitarbeitern<sup>7</sup>, *Zinsser*<sup>8</sup>, *Klinge*<sup>9</sup> und *Clawson*<sup>10</sup> entwickelt. Schon früher war von *Chvostek*<sup>11</sup>, *Menzer*<sup>12</sup>, *Friedberger*<sup>13</sup> und *Weintraud*<sup>14</sup> der Rheumatismus in Beziehung zur Allergie gebracht worden; doch wichen die Anschauungen dieser Forscher in mancher Hinsicht von den heutigen ab.

Schon vor der Entdeckung des *Aschoffschen* Knötchens im Jahre 1904<sup>15</sup>, wurden eine Reihe von Untersuchungen auf Spaltpilze in Gewebsschnitten von Rheumakranken ausgeführt. So fand *Nepveu* im Jahre 1890<sup>16</sup> Mikrokokken und zarte Bakterien in großer Zahl in

einem rheumatischen Knoten des Unterhautgewebes, allerdings in einem zum Teil vereiterten Knoten. *Poynton* und *Paine* (1900)<sup>17</sup> fanden den sog. „*Micrococcus rheumaticus*“ in Schnitten von Unterhautknoten, von Herzklappen, von Herzbeutel und Gaumenmandeln. Von *Wick*<sup>18</sup> wurden staphylokokkenartige Keime in Schnitten von Unterhautknoten beschrieben; aber diese Knoten entstammten Kranken mit chronischer Arthritis und waren zum Teil vereitert. 1931 werden von *Freund* und *Stein*<sup>19</sup> Diplo-Streptokokken in Subcutanknoten von primär chronischer Polyarthritis histologisch festgestellt und auch gezüchtet. Die Keime werden als auffallend vielgestaltig beschrieben; in Gewebsschnitten konnten sie nach Giemsa und Gram dargestellt werden.

Im Gegensatz zu diesen positiven Befunden sind sehr viel negative bekannt geworden. *Aschoff* und *Tawara*<sup>20</sup>, *Coombs*<sup>21</sup>, *Takayasu*<sup>22</sup>, *Bracht* und *Wächter*<sup>23</sup>, *Thalhimer* und *Rothschild*<sup>24</sup>, *Jacki*<sup>25</sup> und *Wäljen*<sup>26</sup> konnten keine Keime in rheumatischen Myokardknoten nachweisen; ebensowenig *Roy*<sup>27</sup>, *Frank*<sup>28</sup>, *Patella*<sup>29</sup>, *Jacki*<sup>25</sup>, sowie *Dawson*, *Olmstead* und *Boots*<sup>30</sup> in subcutanen rheumatischen Knoten. Auch *Pappenheimer* und *von Glahn*<sup>31</sup> berichten über völlig negative Befunde an Mitral- und Aortenklappen<sup>31</sup>, an der rheumatisch erkrankten Aorta<sup>32</sup>, am Vorhofendokard<sup>33</sup> und an verschiedenen Blutgefäßen<sup>34</sup>.

Die Gewebe, an denen die oben ausgeführten Untersuchungen gemacht wurden, stammten von Kranken, die ziemlich lange nach dem Beginn des Rheumatismus gestorben waren, und bei denen zur Zeit des Todes die rheumatischen Schäden ziemlich weit fortgeschritten waren; die positiven Spaltpilzbefunde bezogen sich überhaupt nur auf Hautknoten, meist bei chronischer Polyarthritis.

In der 1. Mitteilung dieser Untersuchungsreihen beschrieb *Klinge*<sup>35</sup> im einzelnen das Gewebsbild ganz frischer, rheumatischer Schäden, in denen es noch nicht zur Entwicklung von *Aschoffschen* Knötchen gekommen war, und es schien deshalb von Bedeutung, diese „rheumatischen Frühinfiltrate“ systematisch auf Spaltpilze zu untersuchen; es wäre ja möglich, daß krankmachende Keime in *frischen* rheumatischen Herden nachzuweisen wären und im voll entwickelten Granulom nicht mehr, eine Ansicht, zu der sich auch *Fr. v. Müller*<sup>36</sup> bekannt hat.

Zu diesem Zweck schien das Material von 2 Kranken geeignet, die 17 und 19 Tage nach dem Beginn des fieberhaften Rheumatismus

verstorben waren und bei denen somit die ältesten Herde nicht älter als 19 Tage und sehr viele viel jünger waren. Der erste Nr. 238/29 ist früher<sup>35</sup> ausführlich beschrieben; der 43jährige Mann ist am 17. Tage dem akuten rheumatischen Anfall selbst erlegen. In dem zweiten Fall (Nr. 207/24 H. R. I.) trat der Tod während einer Tonsillektomie ein, am 19. Tage der akuten Krankheit, deren rheumatische Natur durch den Nachweis rheumatischer Infiltrate sichergestellt wurde. Als dritter Fall wurden die Gaumenmandeln von einem Kind mit halbjähriger Krankheitsdauer herangezogen, das an Herzschwäche gestorben war; die mikroskopische Untersuchung ergab das Bild eines rezidivierenden Rheumatismus mit jungen Narben, mit voll entwickelten Granulomen und mit ganz frischen rheumatischen Frühinfiltraten, besonders auch im peritonsillären Gewebe (Fall 404/30)<sup>37</sup>. Ferner wurden noch zwei rheumatische Unterhautknoten untersucht, die 11 und 15 Tage nach ihrem Auftreten operativ entfernt waren, und außerdem noch eine Reihe Hautknoten etwas älterer Fälle.

Jeder Block wurde in Reihen geschnitten; die meisten Schnitte nach Gram gefärbt, nur jeder zehnte nach Hämatoxylin-Eosin, um die rheumatischen Herde genau ansprechen und lokalisieren zu können. Zahlreiche Schnitte von den gleichen Blöcken wurden auch nach Tibor-Pap versilbert und mit GiemsaLösung gefärbt. Wenn an den Präparaten irgendwelche gefärbte Körnchen ausgefallen waren, wurden diese ganz mit der Ölimmersion durchmustert; wenn nicht, wurde starkes Trockensystem (Vergr. 790fach) verwandt und nur an verdächtigen Stellen Ölimmersion.

### *Ergebnisse*

*Herzfleisch* (Fall Nr. 238/29): 144 fortlaufende Serienschnitte werden durchsucht; sie enthalten 49 rheumatische Frühinfiltrate, deren Bild schon früher beschrieben wurde.<sup>35</sup> Unter den 49 Infiltraten wurden 37 vollständig in der Schnittserie getroffen, so daß von diesen jeder Abschnitt untersucht werden konnte. Nur in *einem* einzigen Schnitt wurde an *einer* Stelle eine kurze Kette deutlicher Kokken gefunden; doch erwies sich diese bei genauer Einstellung als unter dem Deckglas und nicht im Gewebe liegend. Nicht ein einziger deutlicher Keim war in irgendeinem der Präparate zu finden, und nur in 4 fand sich etwas, das entfernt an Mikrokokken erinnerte. Diese verdächtigen grampositiven Körnchen lagen in unregelmäßiger Linie und machten am ehesten den



Eindruck von Zellkerntrümmern; wenn auch die Möglichkeit nicht völlig auszuschließen war, daß es sich um teilweise zerstörte Kokken handelt.

Wir benutzten die Gelegenheit der serienmäßigen Durchsuchung von Herzmuskelstücken, die rheumatischen Infiltrate auf ihre Lagebeziehung zu den Blutgefäßen zu prüfen, da hierüber die Meinungen getrennt sind, ob die rheumatischen Granulome stets in Verbindung mit den Blutgefäßwandungen liegen, oder ob dieses keine gesetzmäßigen Beziehungen sind. Wir fanden, daß die meisten Herde in der Gefäßwand ihren Sitz haben oder doch kleine Arteriolen bzw. Capillaren enthielten. Nur in 5 völlig serienmäßig durchgemusterten Infiltraten war kein Gefäß zu erkennen.

*Herzbeutel* (Fall 238/29): 60 Serienschnitte wurden geprüft. Große grampositive und -negative Bacillen und verschiedenartige Streptokokken ließen sich leicht finden; doch lagen sie alle in und auf der oberflächlichsten Lage der Perikardauflagerungen, an den Stellen, die der früheren Oberfläche des Perikards entsprachen, bevor die Stücke zur Bearbeitung herausgeschnitten wurden. Nicht ein einziger fand sich in den tieferen Gewebsschichten. Der Befund ist ohne weiteres als Verunreinigung zu deuten; denn bei der Sektion ist das Herz mit Wasser und mit dem Sektionstisch in Berührung gekommen, wodurch bakterielle Verunreinigungen sich ohne weiteres erklären.

*Mitralklappe* (Fall 238/29): Die 60 untersuchten Schnitte zeigten alle typische Veränderungen, die man bei rheumatischer Klappenentzündung findet, einschließlich kleiner Wärzchenbildungen. Auf zwei benachbarten Objektträgern mit je 5 aufeinanderfolgenden Schnitten fanden sich einige Kokken und diphtheroide Bacillen, dagegen waren alle in der Serie vorhergehender und nachfolgender Schnitte frei von Keimen.

Dies macht es wahrscheinlich, daß die gefundenen Mikroorganismen als Verunreinigung bei der Herstellung der Schnitte aufzufassen sind, zumal es andererseits nicht zu erklären ist, daß die Bakterien auf dem einen Objektträger so häufig sind und auf dem nächsten völlig fehlen, und daß es sich um verschiedenartige Keime handelt. Ein anderer Grund, das Vorhandensein der Keime als Verunreinigung aufzufassen, ist die Tatsache, daß sie sowohl in den frischen Veränderungen gefunden wurden, als auch im Bereich alter Narben und vor allem auch in völlig normalen Partien der Klappen.

*Aortaklappe* (Fall 207/24 H. R. I.): An 166 Reihenschnitten findet sich das Bild einer sehr frischen Endokarditis mit feinsten Wärzchen. Nur in einem Schnitt liegen über 1000 paar Diplokokken nicht weit von der Basis der Wärzchen, in einem Bezirk der etwa  $90 \times 60$  Mikromillimeter groß ist, und keinerlei entzündliche Reaktion zeigt. Genau wie an der Mitralklappe sind alle vorhergehenden und nachfolgenden Schnitte frei von Kokken. Wenn man bedenkt, daß die Schnittdicke nur 5 Mikromillimeter beträgt, so ist damit bewiesen, daß die einzigen gefundenen Streptokokkenhaufen als Kunstprodukt, bei der Verarbeitung des Schnittes hineingebracht, aufgefaßt werden müssen.

*Zunge* (Fall 238/29): In 96 Präparaten mit zahlreichen Frühinfiltraten sind keine Spaltpilze nachzuweisen.

*Kniegelenkskapsel* (Fall 238/29): Zahlreiche, typische, ganz frische Verquellungsherde der Kapsel und des periartikulären Gewebes, sowie die fibrinoiden Auflagerungen und Verquellungen der Synovia zeigen an 30 Präparaten keine Keime.

*Linke Mandel* (Fall 238/29): In 192 Schnitten fanden sich viele, zum Teil große Frühinfiltrate in der Tonsillenkapsel, zum Teil auf die Muskulatur übergreifend. Alle sind frei von Spaltpilzen, auch die leukocytenreicheren Stellen. An einer einzigen Stelle eines Präparates lag eine deutliche Kette von Kokken im normalen Bindegewebe ohne jede Reaktion. Reichlich Mikroorganismen der verschiedensten Art, darunter massenhaft Diplokokken und Streptokokken waren in den Buchten der Mandel zu sehen.

*Rechte Mandel* (Fall 404/30)\*: Es wurden wieder 192 Schnitte durchgemustert mit zahlreichen rheumatischen Infiltraten, darunter sehr viele ganz frische fibrinoide Verquellungsherde. Das Ergebnis hinsichtlich Bakterienbefunde war völlig negativ.

*Hautknoten* (Fälle T. G. und F. A.-H. R. I. 31): 65 Reihenschnitte von 2 Hautknoten, die 11 bzw. 15 Tage nach ihrem Entstehen entfernt wurden, ergaben keinerlei Bakterien in den typisch rheumatisch veränderten Bindegewebsbezirken.

Außer diesen 2 ganz frischen Hautknoten wurden noch 8 andere von 6 verschiedenen Fällen subchronischer und chronischer Rheumatismen untersucht mit dem gleichen negativen Ergebnis.

### *Besprechung der Untersuchungsergebnisse*

Die kritische Betrachtung unserer Untersuchungsergebnisse führt zu dem Schluß, daß im allgemeinen keine Bakterien histologisch nachzuweisen sind, und daß in den wenigen Schnitten, in denen sich Mikroorganismen finden, alles, besonders auch die Ungleichheit der Keime in demselben Fall, und ihre Lagerung im Schnittpräparat dagegen spricht, sie als ursächlichen Faktor für die Gewebsschäden anzusehen. Aber—wie *Bracht* und *Wächter*<sup>23</sup> betont haben, und wie auch *Obern-dorfer* in der Rheumatismusaussprache im ärztlichen Verein München hervorhebt<sup>24</sup>—die Tatsache, daß Spaltpilze in den rheumatischen Infiltraten nicht zu finden sind, schließt nicht aus, daß sie nicht doch von ursächlicher Bedeutung für das Entstehen der Gewebsschäden sind.

Unsere vorliegenden Untersuchungsbefunde lassen bezüglich der Ätiologie folgende Deutungen zu: 1. Ganze Streptokokken sind in erheblich großer Zahl im ersten Beginn der Gewebsschädigung vorhanden gewesen, sind aber vom Gewebe zerstört, bevor es zur Untersuchung kam.

\* Der Fall ist beschrieben in *Virchows Arch.* 279, 25.

2. Ganze Mikroorganismen sind nie in den Herden vorhanden gewesen, die Schädigung ist bedingt durch irgendeine chemische Schädlichkeit oder durch ein Spaltpilzgift.

3. In rheumatismuskranken Menschen, die gegenüber Streptokokken überempfindlich sind, ist die Reaktionsfähigkeit der Gewebe so groß, daß Gewebsschäden schon hervorgerufen werden durch Mikroorganismen in so geringer Zahl, daß sie mikroskopisch nicht gefunden werden können.

4. Es liegt eine Kombination von mehreren dieser Möglichkeiten vor.

5. Streptokokken haben mit dem fieberhaften Rheumatismus nichts zu tun.

Der dritten dieser Möglichkeiten kommt eine besondere Bedeutung für die Theorie zu, die im fieberhaften Rheumatismus den Ausdruck einer Überempfindlichkeit des Körpers gegen Streptokokken sieht. *Birkhaug*<sup>3</sup>, *Kaiser*<sup>39</sup>, *Irvine-Jones*<sup>40</sup>, *Swift*, *Wilson* und *Todd*<sup>41</sup>, *Coburn*<sup>42</sup>, *Derick* und *Fulton*<sup>43</sup> und *Reichel*<sup>44</sup> haben nachgewiesen, daß Rheumatiker auf Einspritzung von in der Hitze abgetöteten Streptokokken oder deren Produkten in die Haut mit einer erheblich stärkeren entzündlichen Reaktion antworten als normale Vergleichspersonen. Ferner haben *Swift* und seine Mitarbeiter den Beweis erbracht<sup>45</sup>, daß es möglich ist, Kaninchen derart mit Streptokokken zu sensibilisieren, daß eine Einspritzung von 0,00 00 01 ccm einer Bouillonkultur dieser Organismen in die Haut eine entzündliche Hautreaktion von der Stärke und Ausdehnung hervorruft, wie sie beim (nicht sensibilisierten) Normaltier bei gleicher Technik erst mit 0,001 ccm der entsprechenden Kultur zu erzielen ist.

Um diese experimentellen Untersuchungsbefunde mit der vorliegenden Studie in Beziehung zu bringen, wurden 4 Kaninchen nach der Methode von *Swift*<sup>46</sup> sensibilisiert und diese Tiere dann zusammen mit normalen Vergleichstieren in die Haut mit titrierten Verdünnungen einer Kultur nicht hämolytischer und nicht hämoglobinbildender Streptokokken eingespritzt. Dann wurde die Anzahl von Keimen, die in jedem Kubikzentimeter der Kultur vorhanden war, bestimmt. So wurde festgestellt, daß die Reaktionsherde der Haut, die beim Normaltier durch Einspritzung von 200 000 Streptokokken in die

Haut hervorgerufen werden, nach Grad und Ausdehnung denen entsprechen, die beim *sensibilisierten* Tier nach Einspritzung von 20 Keimen auftreten. Die so gesetzten Entzündungsherde der Haut, die eine Ausdehnung von  $10 \times 5 \times 0,5$  mm hatten, wurden nach 48 Stunden herausgeschnitten, in Reihen verarbeitet und nach Gram gefärbt. Sorgfältige Durchsuchung der ganzen Reihen hatte das Ergebnis, daß in den von Normaltieren nach Einspritzung von 200 000 Keimen gewonnenen Präparaten nur ganz gelegentlich und teilweise degenerierte Streptokokken zu finden waren. Es versteht sich dann von selbst, daß in den Schnitten der gleich großen geschädigten Hautbezirke von sensibilisierten Tieren, die nur 20 Keime erhalten hatten, auch nicht ein einziger Streptococcus entdeckt werden konnte.

Nimmt man zu diesen Befunden die oben ausgeführten Tatsachen hinzu, daß bei Rheumatikern eine Überempfindlichkeit gegen Streptokokken besteht, so ist anzunehmen, daß die Gewebe dieser Menschen schon auf so kleine Mengen von Streptokokken reagieren können, daß die Keime histologisch nicht nachweisbar sind. Man wird auch mit der Möglichkeit zu rechnen haben, die auch *Fr. v. Müller*<sup>36</sup> erwägt, daß die pathogenen Keime im Gewebe überempfindlicher Tiere schneller zerstört werden, wenngleich ein exakter Beweis dieser Annahme heute noch nicht erbracht ist. Das eine steht aber fest: Die Tatsache, daß Streptokokken in den rheumatisch geschädigten Geweben nicht gefunden wurden, wie wir es in dieser Arbeit berichtet haben, ist kein Beweis dafür, daß sie für die Entstehung der Gewebsschäden belanglos sind.

#### ZUSAMMENFASSUNG

Weit über 1000 Reihenschnitte von rheumatischen Frühinfiltraten in Myokard, Perikard, Endokard, einschließlich Herzklappen, Mandeln, Synovia und Kapsel der Gelenke, Zunge und Unterhautgewebe von mehreren akuten Fällen von fieberhaftem Rheumatismus wurden auf Streptokokken durchsucht. Es wurden keine Keime gefunden, denen eine ursächliche Beziehung zu den rheumatischen Gewebsschädigungen zugesprochen werden konnte.

Diese negativen Ergebnisse jedoch sprechen keineswegs zwangsläufig dagegen, daß Streptokokken die Ursachen der rheumatischen Gewebsschäden sind, besonders, wenn die Theorie zu Recht besteht,

daß dem fieberhaften Rheumatismus eine Gewebsüberempfindlichkeit (Gewebshyperergie) zugrunde liegt; denn es konnte gezeigt werden, daß ausgedehnte Gewebsschädigungen bei sensibilisierten Kaninchen experimentell zu erzielen sind durch Streptokokken in so geringer Zahl, daß sie schon nach 48 Stunden im geschädigten Gewebsabschnitt histologisch nicht mehr nachweisbar waren.

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## CYTOLOGIC STUDIES ON RHEUMATIC FEVER

### I. THE CHARACTERISTIC CELL OF THE RHEUMATIC GRANULOMA

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PLATES 29 AND 30

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Bang, in 1878 (1), described multinucleated giant cells in a rheumatic subcutaneous nodule, but it was not until Aschoff's (2) discovery of the myocardial submiliary node, in 1904, that attention was focused upon the characteristic microscopic appearance of the granulomata in rheumatic fever. Although a number of investigators (3-5) have questioned the specificity of the Aschoff body, and despite the fact that early and late stages of these lesions do not present all the features of the typical granulomata, it is generally conceded that few microscopic lesions are more characteristic of a given disease than are the fully developed Aschoff bodies. It is likewise quite generally accepted that the essential elements of the fully developed nodules are the large basophilic and often multinucleated cells found in them. Because of the uncertainty concerning the nature of these cells the present study was undertaken in an attempt to obtain additional information by examining the living cells supravitaly stained. The terms "Aschoff body cell" and "cell of the rheumatic granuloma" employed in this paper, refer, of course, to the characteristic cells just mentioned, and not to the lymphocytes, plasma cells, and polymorphonuclear leucocytes which also may be present.

The myocardial submiliary nodules do not lend themselves to the supravital method of examination because of their microscopic size and the difficulty of obtaining them in the fresh state. Subcutaneous nodules, on the other hand, are macroscopic and can be easily excised; and, accordingly, they were selected as the material with which to work. It is obviously important for the purposes of this study, therefore, that the essential identity of these lesions and those in the



myocardium be recognized. Clinically, the relationship between the two has been thoroughly established. The modern appreciation of this fact is well illustrated by the statement of Coombs (6), who, in discussing the various rheumatic manifestations, described the subcutaneous nodules as "the most rheumatic of all." The pathologic evidence supporting this relationship is even more convincing, so that almost all pathologists who have studied the subcutaneous nodules have concluded that morphologically and genetically these lesions are essentially the same as those in the myocardium. Only two opinions to the contrary (7, 8) have been encountered in the literature (see Table I), and the convincing evidence in favor of the analogous nature of the granulomata occurring in different parts of the body in rheumatic fever makes logical the use of the subcutaneous nodules as an indirect means of studying the Aschoff body cells in the myocardium.

Table I summarizes the view of different authors concerning the nature or origin of the peculiar large cells which characterize these lesions. As indicated at the head of the table, the opinions recorded in the first column were derived from studies of subcutaneous nodules, while those in the second column were based upon investigations of the myocardial lesions. Only in those instances in which a different origin has been ascribed to the cells in the two lesions has the opinion of a single author been recorded in both columns. While this list makes no pretence at completeness, it does serve to show the lack of agreement among investigators who have expressed a definite opinion.

Grouping the data from the table in another way, one sees that the cell of the rheumatic granuloma has been thought to arise from different sources by various of these authors as follows: from muscle cells by 5, from endothelial cells by 5, from connective tissue cells by 21, and from wandering or fixed phagocytic cells by 19. This last class, referred to in this communication as clasmatoocytes, probably includes all those cells listed in Table I under the names of adventitial cells, macrophages, mononuclear wandering cells, clasmatoocytes, endothelioid cells, endothelial leucocytes, polyblasts, histiocytes, lymphocytoid elements, and epithelioid cells. By using supravital staining, Sabin and her coworkers (44) have shown the true epithelioid cells of the tubercle to differ from the clasmatoocytes, but in the sense in which these terms were used by the authors in Table I, the names probably referred to cells of the same type.

TABLE I

*Views of Different Investigators on the Nature of the Characteristic Cells of Subcutaneous Nodules and Myocardial Nodules*

Date	Subcutaneous nodules	Date	Myocardial nodules
1878	Bang (1)—Connective tissue cells		
1883	Cavafy (9)—Lymphoid cells		
1883	Carvasy (10)—Young connective tissue cells		
1887	Gilly (11)—Embryonic connective tissue cells		
1889	Cheadle (12)—Connective tissue cells		
1895	Futcher (13)—Fibroblasts		
1904	Wick (14)—Epithelioid cells	1904	Aschoff (2)—Leucocytoid elements (adventitial cells)
		1905	Geipel (3)—Connective tissue cells
		1906	Aschoff and Tawara (15)—Lymphocytoid elements and connective tissue cells
		1907	Coombs (16)—Connective tissue cells
		1908	Saigo (17)—Epithelioid and muscle cells
		1909	Bracht and Wächter (18)—Connective tissue cells
		1910	Roy (19)—Connective tissue and mononuclear wandering cells
		1911	Coombs (20)—Either endothelial or connective tissue cells
		1911	Gallavardin (21)—Epithelioid cells
1912	Frank (22)—Connective tissue cells	1912	Fraenkel (23)—Adventitial connective tissue cells
1913	Voelcker (24)—Connective tissue cells	1913	Huzella (25)—Connective tissue cells
1914	Tilp (26)—Endothelial cells	1914	Huzella (27)—Muscle cells
1914	Patella (7)—Lymphatic endothelial cells	1914	Patella (7)—Connective tissue cells
		1917	Langmann (28)—Endothelial or mononuclear wandering cells
1918	Fahr (29)—Connective tissue cells	1919	Aschoff (31)—Connective tissue cells
1919	Jacki (30)—Polyblasts	1920	Mallory (32)—Endothelial leucocytes
		1920	Whitman and Eastlake (33)—Muscle cells

TABLE I—*Concluded*

Date	Subcutaneous nodules	Date	Myocardial nodules
1923	Perkins (34)—Endothelioid cells	1923	Kanatsoulis (35)—Epithelioid cells
1924	Swift (36)—Endothelial cells		
		1925	MacCallum (37)—Clasmatocytes
		1926	Letulle, Bezançon, and Weil (38)— Muscle and connective tissue cells
		1926	Sacks (39)—Histiocytes
1927	Gräff (40)—Adventitial and connective tissue cells		
1928	Symmers (8)—Connective tissue cells	1928	Symmers (8)—Muscle cells
		1929	Clawson (4)—Polyblasts
		1929	Gross, Loewe, and Eliasoph (41)— Histiocyte family
		1930	Klinge (42)—Mesenchymal cells
		1931	Donaldson (43)—Histiocytes

### *Material and Technique*

It is especially important that the rheumatic nature of the nodules used in this study be firmly established because of the occurrence of somewhat similar lesions in other diseases. The so called juxta-articular nodules of syphilis, yaws, acrodermatitis chronica atrophicans, and scleroderma may be mentioned among these, although it is most improbable that they would be mistaken for rheumatic lesions. For accounts of them the reader is referred to the papers of the Harvard African Expedition (45), of Jessner (46), and of Hopkins (47). Subcutaneous nodules have likewise been reported in patients with infectious arthritis, by Hawthorne (48), Coates (49), Dawson and Boots (50), and others; as some of these authors have pointed out, however, this probably indicates merely the close connection between this disease and rheumatic fever. Finally, Coates and Coombs (51) have described similar lesions occurring in a patient with endocarditis lenta, although the nature of the disease apparently was not unquestionably established since there is no mention of a blood culture in the report and the patient, who had previously had rheumatic fever, recovered.

The present study is based upon an investigation of eleven subcutaneous nodules from ten patients with rheumatic fever, six of whom were from the Hospital of The Rockefeller Institute, while the remaining four were from the Children's Medical Service of Bellevue Hospital. From nine the material was obtained at biopsy, while from one it was obtained at autopsy within  $\frac{1}{2}$  hour of the patient's death. All the patients had typical rheumatic fever with carditis and polyarthritis in addition to the subcutaneous nodules, and all were between the ages of 5 and 22 years. The blood Wassermann reaction was negative in the six patients in

whom the test was performed, while in the other four, whose ages ranged from 5 to 12 years, there were no stigmata of congenital lues. In no case was there reason to suspect bacterial endocarditis and, in the one patient who died, autopsy revealed no evidence of that disease.

The control material comprised: skin and subcutaneous tissue from a patient with erythema nodosum and rheumatic heart disease, a piece of subcutaneous tissue and deep fascia from a boy with rheumatic subcutaneous nodules, an "ephemeral node" from a case of bacterial endocarditis, a bit of triceps tendon and deep fascia from the elbow of a nephritic patient, and numerous scrapings of the deep fascia, tendons, periosteum, and endothelial linings of blood vessels of rabbits and dogs.

Preliminary to excision in two of the cases, material was obtained by inserting a needle of wide caliber into the tumor mass and then withdrawing bits of tissue through it by means of a dental broach. This method, described by Forkner (52), is satisfactory for lymph nodes and other friable tumors, but was inadequate for obtaining a sufficient amount of material from subcutaneous rheumatic nodules. In both cases in which this method was employed, however, a few cells were obtained which could be identified from the previous experience with excised lesions.

Each of the excised nodules was cut in half and one piece was immediately placed in Zenker-acetic solution or in formol for the preparation of serial sections. The other half was freed from non-granulomatous material and was then scraped with a sharp scalpel. The small amount of material thus obtained was treated as follows:—

1. Films were made on clean glass slides by mixing the scrapings in a drop of physiologic saline, which was then allowed to dry. They were fixed in Zenker-acetic solution 15 minutes, washed in water for several hours, and finally stained in several ways, the most satisfactory of which was the malachite green-acridine red method of Hitchcock and Ehrich (53).

2. Supravitality stained preparations were made according to the technique described by Sabin (54). Dried films of neutral red and Janus green were prepared.<sup>1</sup> The scrapings from the nodules were mixed in a drop of physiologic salt solution upon cover-slips which were then inverted upon the prepared slides and

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<sup>1</sup>The dyes used were those of the National Aniline and Chemical Company, New York. A stock solution of neutral red was made by dissolving 125 mg. of the powdered dye in 50 cc. of neutral absolute alcohol; and that of Janus green (diazine green) by dissolving 125 mg. in 62.5 cc. of absolute alcohol. From the stock neutral red a dilute solution was made by adding 50 drops to 10 cc. of absolute alcohol. All these solutions keep very well separately, but when neutral red and Janus green are mixed in solution they deteriorate rapidly; hence the combined stain for the preparation of the slides was made just before use. This was done by mixing thoroughly 2 drops of the Janus green solution in 3 cc. of the dilute neutral red. Carefully cleaned slides were then flooded with this mixture and were dried above a small flame so that the dyes remained as a thin, even film.

sealed with vaseline. In some instances the scrapings were mixed in a drop of dilute saline solution of neutral red or Janus green upon the prepared slides in order to increase the amount of available dye. These preparations were immediately examined under the oil immersion lens in a warm chamber at 37°C.

In one instance this procedure was altered to ascertain whether the intravital injection of particulate matter would lead to phagocytosis by the cells of the nodule. A sterile 50 per cent suspension of India ink in physiologic saline was injected into and around the nodule. Biopsy was performed 40 hours later and the excised material was subjected to the procedures already outlined.

### RESULTS

All the supravitality stained preparations showed small masses of tissue composed of many cells lying in a fibrillar meshwork, and of wavy fibrils such as occur in similar preparations of tendons or deep fascia. Only at the margins of these masses, however, could the cells be clearly distinguished. Lying between the bits of tissue were large numbers of these same cells which, because of their isolated positions, could be more accurately studied. Of these, the great majority were of the type illustrated in Fig. 1 *A*, but all transitions were seen ranging from small cells about the size of intermediate lymphocytes to spindle-shaped cells and large, multinucleated giant forms. In addition, erythrocytes were always present, while in several preparations there were a few small lymphocytes, monocytes, and clasmotocytes. Occasional cells were encountered which had the appearance of plasma cells as described by Miller (55).

The predominating cell in supravitality stained preparations was from 15 to 20 microns wide by 20 to 30 microns long. The small cells, however, were sometimes only 8 x 15 microns in diameter, while the multinucleated cell in Fig. 3 measured 32 x 77 microns. The shape was usually oval, but many of the cells had pointed processes at one end which were often at a sharp angle to the rest of the cell, while the long, spindle-shaped elements shown in Fig. 1 *B*. were not uncommon.

The cell membrane in freshly studied preparations was very indistinct, but was more definite in those kept in the ice box for 48 hours. The cytoplasm had a coarse, ground glass appearance, and its pale yellowish gray color showed it to be slightly basophilic. The nucleus was oval and large, almost filling the small cells, but occupying relatively less of the larger ones. In sharp contrast to the vague cell

outline, the nuclear membrane was extremely distinct. The nuclear background had almost the same appearance as the cytoplasm, but the ground glass markings were coarser and the basophilia slightly greater. Indeed, the nucleus and cytoplasm were so similar that, if it had not been for the sharply outlined nuclear membrane, it would have been difficult to distinguish between them. One or two nucleoli were usually present.

Definite mitochondria were never seen, although in a few cells a faint suggestion of minute, pale blue dots was noted. A striking contrast to this was observed in the case of the lymphocytes present in small numbers in many of the preparations; in these cells the deep blue mitochondria were always easily seen, especially in scrapings stained with an additional drop of dilute saline solution of Janus green. The study of fixed material stained by Altmann's method (56) also failed to reveal mitochondria.

The failure of the cells to take up neutral red was their most striking characteristic. In a few cells two to four small, pale pink dots were seen which faded after about 30 minutes. The great majority, however, showed none of the dye at any stage of the examination, and there was a complete lack of phagocytosis even in preparations exposed to neutral red for 30 days at ice box temperature. A few small refractive bodies were seen in almost all the cells, but they seemed never to be stained by the dye.

Fig. 1 is a drawing of some of the cells from one nodule. At *E* can be seen the edge of one of the large masses of fibers and cells which were always present in the scrapings. At *A* is shown a cell of the type which comprised about 80 per cent of all those in the preparations. The coarsely granular character of the cytoplasm is better illustrated in Fig. 2, a microphotograph of a similar group of cells; but the drawing depicts very well the sharply outlined nuclear membrane, the refractive bodies in the cytoplasm, and the nucleoli. In Fig. 1 at *B*, *C*, and *D* are shown variations from this predominant type. Of these the small cell at *C* probably represents merely an earlier stage, while the others show definite alterations in form. The two cells labeled *B* show transition toward the long spindle-shaped elements which resemble fibrocytes, while that marked *D* is a giant cell with two nuclei. The best example of the latter type of cell, however, is seen in the microphotograph (Fig. 3), which likewise depicts very clearly the vague cell boundary and the very distinct nuclear membranes. The scrapings from this nodule contained a few clasmatocytes and two of these are shown for contrast (Fig. 1 *F*). In these clasmatocytes the definite cell boundary, the vague nuclear borders, and

especially the large number of neutral red-stained bodies serve to differentiate them very sharply from the characteristic cells of the rheumatic granuloma.

As has been mentioned, scrapings of all the nodules were fixed in Zenker-acetic solution and were stained by the malachite green, acridine red method. Such preparations showed cells having the same staining characteristics as those in the usual paraffin sections. The cytoplasm took the red dye with varying intensity, apparently depending upon the age, size, and state of preservation of the cell. The dark blue and sharply outlined nuclear membrane contrasted sharply with the pale blue of the nuclear background, and against this the deep red or blue nucleoli stood out plainly.

The study of supravitality stained scrapings from the nodule into which India ink had been injected was particularly interesting. Most of the cells were exactly like those described above, but a few contained particles of carbon as shown by a differential count of 200 cells, of which 95 per cent contained no India ink, 3 per cent contained 2 to 3 small particles, while in the remaining 2 per cent the carbon was present in fair amount. Even these phagocytic cells, however, failed to stain with the neutral red. Study of sections of this nodule bore out these results. A moderate amount of intercellular carbon was scattered diffusely through the nodule and surrounding tissue and there were many polymorphonuclear leucocytes, a large number of which contained ink granules. In the perinodular tissue were many typical clasmatoocytes, completely filled with particles of carbon. In the characteristic nodule tissue, on the other hand, only a small percentage of the granuloma cells contained a few small particles, and typical clasmatoocytes were entirely lacking in spite of the presence of much unphagocytosed carbon.

While all the rheumatic nodules consistently yielded large numbers of the characteristic cells illustrated in Figs. 1 and 2, nothing comparable was found in any of the control material. A few elongated cells occurred in scrapings of normal rabbit tendons or deep fascia and of similar tissue taken from patients who had died of other diseases. These were similar in many respects to cells of the type shown in Fig. 1 B, but were very infrequently encountered and the rounded forms and giant cells were never found. The piece of deep fascia from a

patient with rheumatic nodules which was mentioned among the control material, deserves further comment. This patient had definite nodules in areas not suitable for excision and in addition a very small one situated over the left patella. An attempt was made to excise the latter and the material obtained was immediately subjected to the procedures previously outlined but no cells of the rheumatic granuloma type were found in supravital stained preparations. Subsequent examination of the paraffin sections of this material, however, revealed only normal connective tissue, and hence it was obvious that the nodule had been missed at the time of biopsy.

#### DISCUSSION

The success attained in identifying the cells in the lesions of tuberculosis and syphilis by means of the supravital staining method led to its employment in this study of the rheumatic granulomata. Sacks (39) had already suggested that the subcutaneous nodules would lend themselves to such an investigation; and Cecil (57) used vital staining to identify cells in the myocardial lesions of rabbits which had been injected intravenously with streptococci. He found these cells to be macrophages, but since he believed the experimentally produced lesions to differ from Aschoff bodies, the investigation did not cast much light on the nature of the cells in the latter.

As indicated in Table I, a number of possible origins have been suggested for the peculiar cells of rheumatic granulomata, and each of these will be considered.

First, it is fairly certain that fragments of cardiac muscle fibers lying in Aschoff bodies may sometimes have the appearance of multinucleated giant cells, especially when the fibers are regenerated and have been cut transversely. In all probability, however, this is not the source of the true Aschoff body cells; indeed, if one agrees with the generally accepted view that the granulomata in the cardiac valves and auricular endocardium are analogous to those in the myocardium (58), it is obvious that the characteristic cells of these lesions cannot be dependent upon muscle fibers for their origin.

The possibility that the cells of the rheumatic granuloma arise from endothelium is suggested by the great vascularity and extensive endothelial swelling and proliferation in histologic sections of many



subcutaneous nodules. On the other hand, such changes are not present in all nodules; and, conversely, similar endothelial swelling may occur in any area containing newly formed capillaries. Moreover, careful search of serial sections of myocardial Aschoff bodies sometimes fails to reveal the presence of any vessels in or near the lesions (59). Hence the close resemblance of the swollen endothelial cells to the typical Aschoff body cells, in ordinary histologic sections, is merely suggestive of a relationship between them. Unfortunately, the method employed in the present work has not given, as yet, a definite answer to the question thus raised. Endothelial cells obtained by scraping vessels differ greatly in appearance from the cells of the rheumatic granuloma, in that they are more spindle-shaped and lie in sheets; but those free elements in the circulating blood which Sabin and Doan (60) have called desquamated endothelial cells bear a close resemblance to cells of the type illustrated in Fig. 1 B.

The great majority of authors listed in Table I selected connective tissue cells or those of the clasmatoocyte group as the source of the elements of the rheumatic granulomata. These two views can be conveniently discussed together, since the latter elements were probably included in the broad term "connective tissue cells" as it was employed by pathologists up to recent years. Indeed, opinions concerning the origin and nature of the Aschoff body cells hinge largely upon the nomenclature of the different elements of the normal and pathologic loose connective tissue. The omentum serves as an excellent example of such tissue, in which one can identify three chief cellular components: (a) the true adventitial cells of Marchand, which are actively phagocytic and belong to the group called clasmatoocytes in this communication; (b) the fibrocytes; and (c) the primitive mesenchymal cells, which have a great capacity for further differentiation. The chief distinguishing feature of the first of these types, in supravitality stained preparations, is a marked ability to take up neutral red. The present investigation has shown that the typical cells of subcutaneous rheumatic nodules, on the contrary, are incapable of this; hence they cannot be clasmatoocytes. This same characteristic tends to exclude the fibrocytes as the source of the cells in question, since Carrel and Ebeling (61) and others have shown that they, too, take up the dye in moderate amount, especially when actively dividing. In the scrap-

ings here examined there were some cells (Fig. 1 *B*) which in shape suggested fibrocytes. The characteristics of all the granuloma cells, however, including these spindle-shaped elements, were those of young connective tissue cells less differentiated than the fibrocytes. This study, therefore, points strongly to the primitive mesenchymal elements as the source of the cells under investigation.

In connection with the fact that both connective tissue and endothelium have been suggested as the source of the cells in rheumatic granulomata, the views of Klinge are of particular interest. This author (42) has interpreted rheumatic fever as a disease essentially of the mesenchyme. He believes that the characteristic cells arise chiefly from mesenchymal elements normally present in connective tissue, but, in addition (62), that perimysial cells and endothelial cells lining capillaries and lymph spaces may also take part, since they are of mesenchymal origin. Recently, Rinehart (63) has summarized the data favoring the view that capillaries and lymph vessels arise *in situ* from undifferentiated mesenchyme, and has presented additional evidence that the endothelial cells are only slightly differentiated from the primitive mesenchymal elements. These theories, although still debatable, offer a possible explanation of the apparent participation of both connective tissue and endothelial cells in the formation of some of the lesions; and it may be that, although the primitive mesenchymal cells respond most readily to the stimulus of rheumatic fever, other cells, not too highly differentiated, may also take part.

The microscopic lesions of tuberculosis, syphilis, and rheumatic fever are all composed of unit cells which bear a strong resemblance to one another in ordinary histologic sections and which probably all arise from the undifferentiated mesenchymal cells. It is of interest, therefore, to compare these unit cells in their reaction to the supravital dyes, and at the same time to note the different modes of development of the primitive mesenchymal elements in these three diseases. Sabin, Doan, and Forkner (64) have shown that in experimental tuberculosis there is, early, a great increase in the number of these undifferentiated cells, which rapidly evolve into monocytes and epithelioid cells (the latter term being restricted, here, to the typical elements of the tubercle characterized by the neutral red rosette which has been described by the same authors). By development in a different man-

ner under the influence of other stimuli it is probable that the same primitive elements become clasmatoocytes (65), distinguished from the epithelioid cells by their more active phagocytosis and the different manner in which the engulfed dye is distributed through the cytoplasm. Following the work of Sabin and her coworkers, Morgan (66) showed the characteristic and predominating cell in experimental syphilis to be the clasmatoocyte, although Pearce and Rosahn (67) found chiefly monocytes in the early lesions and clasmatoocytes later. In the present study of rheumatic nodules, on the other hand, monocytes and clasmatoocytes were only rarely encountered, and epithelioid cells, never. Through the kindness of Dr. Sabin, the author had the opportunity of studying material from guinea pigs injected with tuberculo-fatty acid; and, in very early lesions, many cells were found identical in every respect with the rheumatic granuloma cells of the type illustrated in Fig. 1 A. Even in these early lesions, however, the majority of cells had already begun to assume the staining characteristics expected in the cells of the tubercle. In rheumatic fever, as in tuberculosis, the number of primitive mesenchymal cells apparently increases markedly and they evolve into mono- and multinucleated forms. The cells of the rheumatic granuloma differ from those of the tubercle and the gumma, however, in their inability to stain supravitaly with neutral red, and they continue to have the characteristics of undifferentiated, young, connective tissue cells at all stages of their development.

It has been recorded that some of the granuloma cells of the nodule into which India ink had been injected prior to biopsy contained particles of carbon. This probably indicates that they can acquire the function of phagocytosis when acted upon by proper stimuli. Such developmental potentialities would be entirely in keeping with the view that the cells are relatively undifferentiated functionally. Transition cells such as those illustrated in Fig. 1 B are, perhaps, further evidence that the elements of the rheumatic granuloma can change into other types, in this instance, into fibrocytes; and it is suggested that this latter transformation may explain the great tendency of these lesions to produce scar tissue.

#### SUMMARY AND CONCLUSIONS

Scrapings of subcutaneous nodules from ten patients with rheumatic fever were examined microscopically after being stained with supra-

vital dyes. From the uniform results obtained, the following conclusions have been drawn.

1. Supravital staining of cells from these lesions gives information unobtainable with ordinary histologic methods.

2. The scrapings show a great predominance of certain cells almost entirely devoid of phagocytic power and not characterized by the reactions with neutral red which distinguish monocytes, epithelioid cells, and clasmatoocytes. Hence they differ from the essential cells of the lesions of tuberculosis and experimental syphilis. These differences are probably of a functional and developmental rather than of a genetic nature.

3. The cells probably arise from the undifferentiated mesenchymal elements of loose connective tissue, although it is possible that endothelial cells take part in their formation in some instances.

4. Since there is little doubt that the subcutaneous rheumatic nodules are pathologically identical with rheumatic granulomata elsewhere in the body, these conclusions are considered applicable also to the Aschoff body cells of the myocardial submiliary nodules.

It is a pleasure to acknowledge the advice and assistance given by Dr. Florence R. Sabin throughout the course of this study. The author also wishes to thank Dr. Lucy Porter Sutton for permission to study the cases at Bellevue Hospital, and Dr. D. A. De Santo for performing the biopsies at Bellevue Hospital.

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## EXPLANATION OF PLATES

### PLATE 29

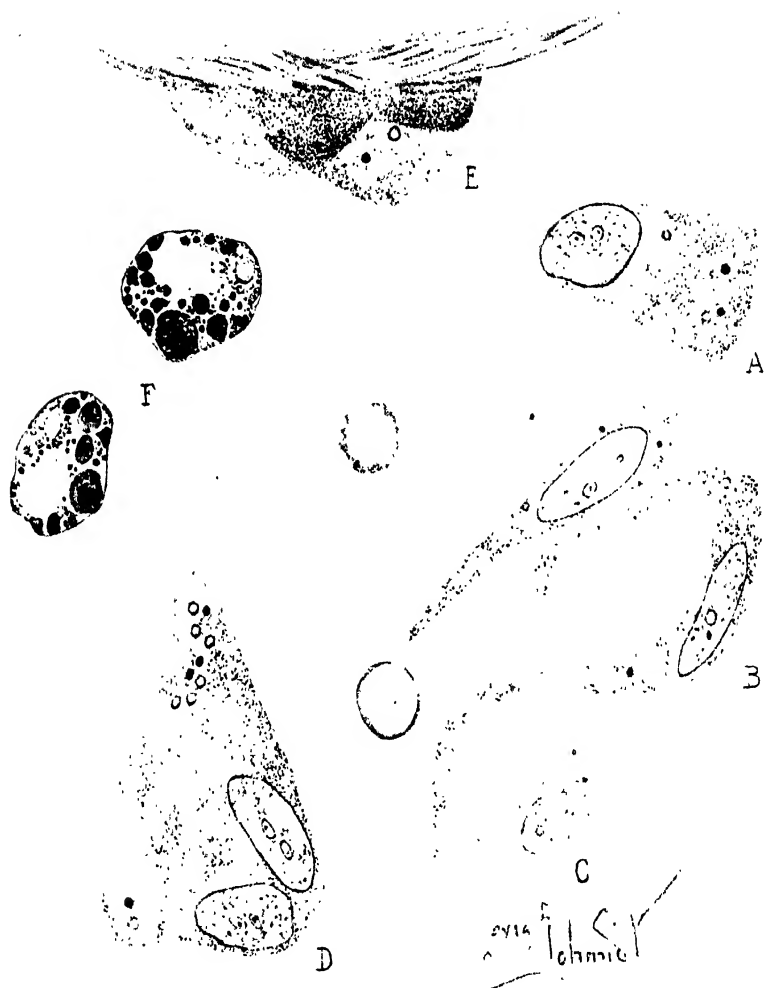
FIG. 1. Drawing of cells from a subcutaneous rheumatic nodule. At *A*, *B*, *C*, and *D* are shown rheumatic granuloma cells, while at *E* is seen the edge of a small mass of tissue. *A* represents the predominant type of cell, *B* the long, spindle-shaped elements, *C* a small cell, and *D* a multinucleated form. The dark and light gray bodies in the two clasmatoocytes (*F*) were actually dark and light shades varying from red to orange in the original. Two erythrocytes are included for comparison of size. Stained supravitaly with neutral red and Janus green.  $\times 1,700$ .

### PLATE 30

FIG. 2. Microphotograph of a collection of cells of the type which predominated in the scrapings from subcutaneous nodules. In the group at the bottom of the figure one of the cells is superimposed upon another. Stained supravitaly with neutral red and Janus green.  $\times 1,700$ .

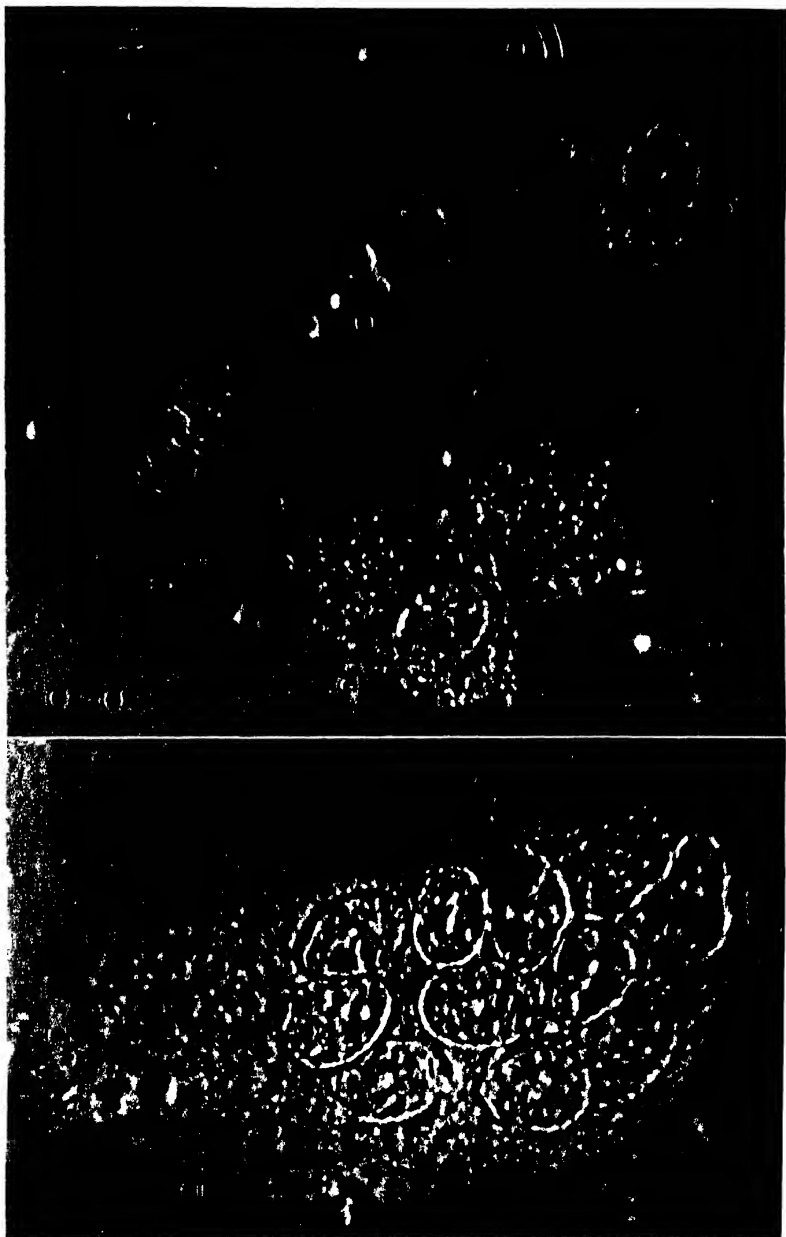
FIG. 3. Microphotograph similar to that in Fig. 2, but showing a giant cell containing eleven nuclei. The granular cytoplasm, nucleoli, vague cell boundary, and very distinct nuclear membranes are particularly well shown in this photograph.  $\times 1,700$ .











Photographed by Louis Schmidt

(McEwen: Cytologic studies on rheumatic fever. 1)



## REACTION OF RABBITS TO STREPTOCOCCI: COMPARATIVE SENSITIZING EFFECT OF INTRACUTANEOUS AND INTRAVENOUS INOCULA IN MINUTE DOSES

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It has been demonstrated that chronic low-grade infection, or numerous localized foci of inflammation induced in the rabbit by repeated minute inoculations with relatively avirulent (green or indifferent) streptococci, evoke a state of hypersensitivity to the bacteria (1, 2). This hypersensitivity may be demonstrated by hyperreaction to minute doses injected intracutaneously, by an ophthalmic reaction following application of the bacteria to the scarified cornea, and by death in from 24 to 48 hours after intravenous doses tolerated by normal animals (2). Suitable preliminary intravenous inoculations result in another type of response; the characteristic hyperergic reactions are not induced; skin lesions are smaller than in normal animals and quickly assume a hard nodular character without much macroscopic evidence of edema; ophthalmic tests are negative; the responses are those of immune animals (3).

It is possible that these differences in response of animals to the two modes of preliminary inoculation might be conditioned by a small antibody content in the animals inoculated intracutaneously compared with a larger amount in those inoculated intravenously. The induction of high degrees of bacterial hyperergy with repeated small inocula of streptococci suggested comparing the effect of similar small doses given intravenously; for from both routes a low antibody production might be expected because of the small amount of antigen introduced. Thus, the question as to whether these differences in the reactive state of the tissues might be the result of suitable doses of antigen, rather than of its site of action, focal or general, seemed open to further

investigation; and the following experiments were designed to throw additional light on this subject.

### *Method*

For the first experiment a vaccine was made from a hemolytic streptococcus, Strain S 43, originally isolated from the throat of a patient with measles. This organism killed rabbits of 2,500 gm. within 24 hours following intravenous inoculation with 1.5 cc. of broth culture. It was preserved in the frozen and dried condition (4) from which fresh blood broth stocks were prepared monthly. Fresh vaccines were made each week from the centrifugate of 20 hour tryptic broth culture, resuspended in Ringer's solution, and heated at 56° for 35 minutes. The purity of the culture and sterility of the vaccines were suitably controlled.

In the second experiment, living cultures of *Streptococcus viridans*, Strain V 110 A, originally isolated from a rheumatic subcutaneous nodule, were used. Cultures were preserved and grown in the manner described for Strain S 43; and fresh cultures were used each day, with microscopic and cultural control of purity.

Dilutions for intracutaneous inoculations were prepared in Ringer's solution, in the first experiment from suspensions of heat-killed centrifugate made to the same volume as the original culture, and in the second experiment directly from the cultures. The volume injected was 0.1 cc. in all instances: an inoculation of  $10^{-1}$  cc., therefore, consisted in 0.1 cc. of original vaccine or culture,  $10^{-2}$  cc. of 0.1 cc. of a 1:10 dilution, and  $10^{-3}$  cc. of 0.1 cc. of a 1:100 dilution. The size of lesions produced is recorded in terms of cubic millimeter volume, estimated from measurements of two diameters and the height of each lesion, as described elsewhere (2).

For the ophthalmic reaction (2) cultures were concentrated 50:1 in Ringer's solution, and 0.3 cc. of suspension was instilled in the conjunctival sack after corneal scarification. In the first experiment the suspension was heat-killed; in the second, living cultures were used.

The serum agglutinin titers were determined in the following way: a large volume of tryptic digest broth (1,500 cc.) was inoculated with 50 cc. of an 18 hour culture and grown at 37°C. for 7 hours. After centrifugation the sediment was concentrated (100:1 or more) in plain broth to make a suitable suspension. Reactions were read after 2 hours' incubation at 56°C. These organisms yielded unstable suspensions unless such precautions were taken.

### EXPERIMENTAL

*Experiment 1.*—As indicated in Chart 1, quantities of  $10^{-2}$  cc. and  $10^{-3}$  cc. of hemolytic streptococcus vaccines were given daily for 41 days. These doses were injected intracutaneously at two sites in each of the six rabbits of Group A, and a similar amount was given intravenously to the five rabbits of Group B.

At intervals of 1 week the lesions in the skins of Group A were measured. As the chart shows, a fair degree of sensitivity was demonstrable in this group by the 22nd day; but it diminished later and rose again towards the end of the experiment. Such fluctuations are not unusual and occurred also in the second experiment.

On the 45th day the comparative sensitivity of the two groups was tested by intracutaneous inoculations with  $10^{-4}$  cc.,  $10^{-5}$  cc., and  $10^{-6}$  cc. of vaccine. These small doses were employed in order to minimize the possible sensitizing

TABLE I  
*Experiment 1*  
*Comparison of Groups on 45th Day*

Rabbit No.	Cutaneous reaction		Ophthalmic reaction*	Agglutinations						
				1:10	1:20	1:40	1:80	1:160	1:320	1:640
25-69	Group A Intracutaneously sensitized	±	+	±	±	±	—	—	—	—
25-70		±?	±	±	±	—	—	—	—	—
25-71		±?	+	±±	±±	±	—	—	—	—
25-72		+	±±	±	±	—	—	—	—	—
25-73		±	±	±	±	±	—	—	—	—
25-74		+++	++++	±	±	±	—	—	—	—
25-81	Group B Intracutaneously immunized	—	+	±	±	—	—	—	—	—
25-82		—	±	+	±	±	—	—	—	—
25-84		—	—	±±	±±	++	++	±	±	—
25-85		—	—	±	—	—	—	—	—	—
25-86		—	—	±±	±±	±	±	—	—	—

Negative reaction indicated: —.

Increasing degree of reaction indicated: ± to ++++.

\* Right eyes tested at this time.

effect of intracutaneous inocula in Group B in later periods of the experiment. The results, summarized in Table I, showed Group A to be much more sensitive, both in cutaneous and ophthalmic reactions. The agglutinin content of the sera of the animals in both groups was quite comparable, with possibly slightly more in Group B.

From the 51st to the 69th days, larger doses, usually of  $10^{-1}$  cc. and  $10^{-2}$  cc. were given daily. On the 70th day comparative titrations were again performed, but with inocula ranging from  $10^{-1}$  cc. to  $10^{-5}$  cc. At this time a group of four rabbits previously untreated (Group C) was introduced into the experiment. The results are indicated in Chart 1 and Table II.

Five animals of Group A responded with very large cutaneous reactions,

Effect of repeated small inocula of hemolytic streptococcus vaccine (Strain S 43). Group A (6 rabbits) intracutaneously, Group B (5 rabbits) intravenously, Group C (4 rabbits) normal.

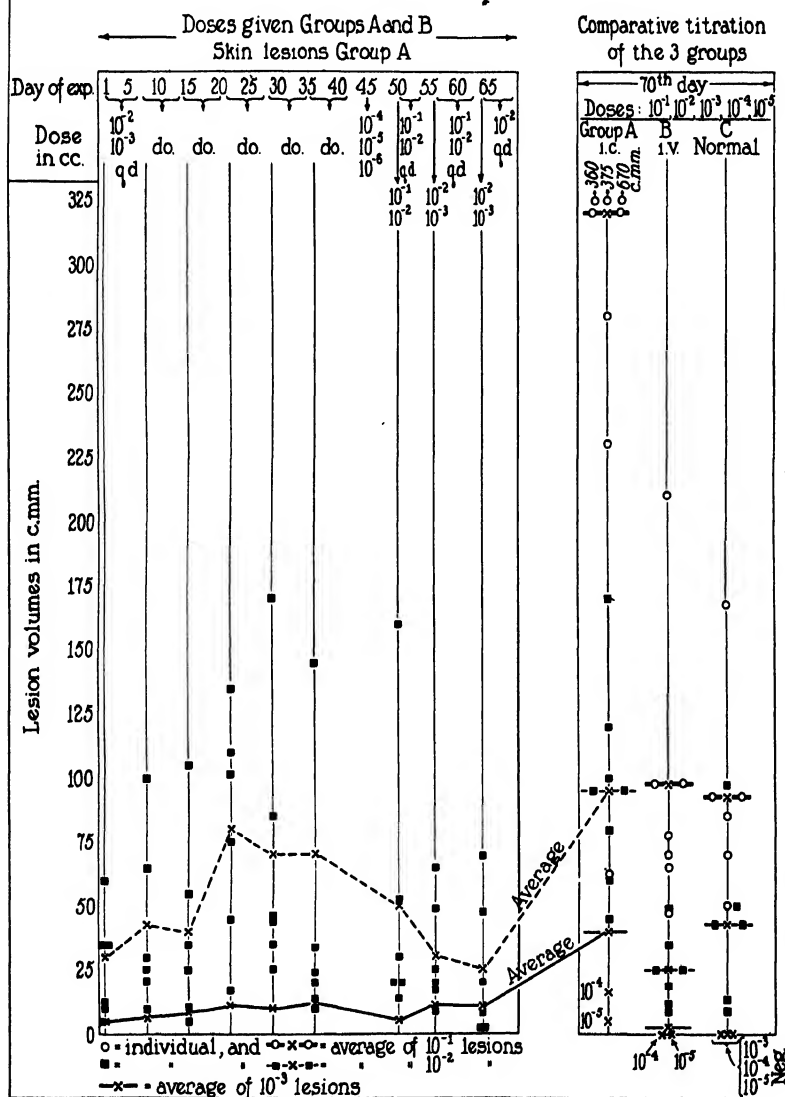


CHART 1

those of Group B with distinctly smaller reactions, but these, on the other hand, averaged very slightly larger than in Group C, normals, inoculated the first time with comparable doses of vaccine. The lesions in Group B were, however, hard and nodular and had very little edema about them at a time when the lesions in Group A were very red and edematous. The ophthalmic reactions indicated that the corneae of the intravenously immunized animals were less sensitive to the irritating action of the vaccine than were those of Group A. The relatively

TABLE II  
*Experiment 1*  
*Comparison of Groups on 70th Day*

Rabbit No.	Cutaneous reaction	Ophthalmic reaction*	Agglutinations							
			1:10	1:20	1:40	1:80	1:160	1:320	1:640	Control
25-69	Group A Intracutaneously sensitized	++++	++	+	±	±	±	-	-	-
25-70		+	±	±	+	±	±	±	-	-
25-71		++++±	±	±	±	±	±	-	-	-
25-72		+++	±	±	+	±	±	-	-	-
25-73		++±	±	±	±	-	-	-	-	-
25-74		++++	++++	±	+	±	±	±	-	-
25-81	Group B Intravenously immunized	±	±	+	±	±	±	-	-	-
25-82		++	-	+	±	±	-	-	-	-
25-84		±	-	++	++	++	++	++	+	-
25-85		±	-	+	±	-	-	-	-	-
25-86		±	-	±	±	±	-	-	-	-
26-55	Group C Normal	±	±	±	±	±	-	-	-	-
26-56		±	±	±	-	-	-	-	-	-
26-58		±	±	±	±	-	-	-	-	-
26-59		+	-	±	±	-	-	-	-	-

\* Left eyes tested at this time.

low serum agglutinin titer was about the same in Groups A and B; but slight reactions were obtained with normal sera.

*Experiment 2.*—As summarized in Chart 2, the animals of Group A were sensitized as follows: After initial multiple inocula ranging from  $10^{-1}$  cc. to  $10^{-5}$  cc. of broth culture of *Streptococcus V 110 A*, a single dose of  $10^{-3}$  cc. was given daily for 16 days; and on the 2nd, 7th, and 14th days the degree of sensitivity was tested with multiple inocula. The animals of Group B received the same doses of culture intravenously. The results of their comparative ophthalmic sensitivity and the agglutinin content of their blood sera on the 18th day is shown in Table III.



The rabbits in Group A were distinctly sensitive at this time, as indicated both by the cutaneous and ophthalmic reactions. Group B and Group C were not injected intracutaneously on this day; but the ophthalmic reaction performed in all three groups indicated decided sensitivity in Group A; two very slight reactions occurred in Group B, and none in Group C. The serum agglutinin titer was about the same in Groups A and B, and was distinctly increased in comparison with the normal group.

TABLE III  
*Experiment 2*  
*Comparison of Groups on 18th Day*

Rabbit No.	Cutaneous reaction	Ophthalmic reaction*	Agglutinations							
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	Control
26-91	Group A Intracutaneously sensitized	++++	++++	±	±	-	-	-	-	-
26-92		+++	++++	++	++	±	-	-	-	-
26-93		+++	++++	+	+	±	-	-	-	-
27-31		++++	++++	++	++	++	+	-	-	-
26-95		+++	++++	++	++	++	±	+	±	-
26-96		++	+	+	-	-	-	-	-	-
26-97		+++	+++	+	±	±	-	-	-	-
26-98	Group B Intravenously immunized	±	++	++	++	+	+	±	-	-
26-99		-	++	++	+	±	-	-	-	-
27-00		±	++	++	+	±	±	-	-	-
27-01		-	++	++	±	+	-	-	-	-
27-02		-	++	±	±	-	-	-	-	-
27-03		-	++	++	++	+	-	-	-	-
27-04		-	++	++	++	±	+	-	-	-
27-06	Group C Normal	-	±	-	-	-	-	-	-	-
27-08		-	-	-	-	-	-	-	-	-
27-09		-	-	-	-	-	-	-	-	-
27-05		-	-	-	-	-	-	-	-	-
		Not tested this date								

\* Right eyes tested at this time.

Subsequently small inocula were given only on the 23rd, 28th, and 34th days; but, as indicated in Chart 2, skin sensitivity increased in Group A until the 28th day, then fell off somewhat when comparative tests were repeated in all groups on the 44th day. The results are summarized in Chart 2 and Table IV.

The skin lesions were large and edematous in Group A, and much smaller in Groups B and C, with a slight, but distinctly greater and more nodular response in Group B than in the normal group (C). (In the latter group one

Effect of repeated small inocula of green streptococcus culture (Strain V 110 A) Group A (7 rabbits) intracutaneously, Group B (7 rabbits) intravenously, Group C (3 rabbits) normal.

Doses given Groups A and B

Skin lesions Group A

Comparative titration of the 3 groups

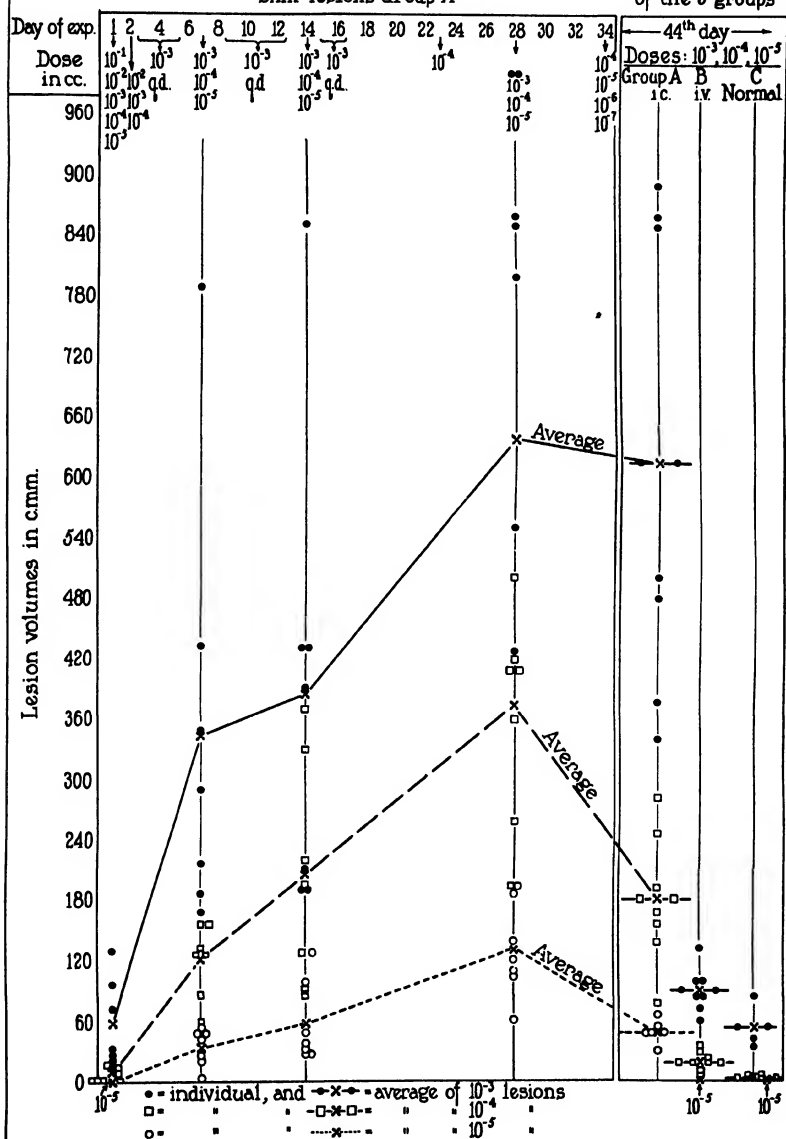


CHART 2

rabbit had died of an intercurrent infection, leaving only three for this last titration.) Again, a distinct increase in serum agglutinins was demonstrated in Groups A and B. Furthermore, the titer was, in general, higher in the intravenously inoculated group (B) than in the group receiving the injections intracutaneously (Group A), but the cutaneous reactions seemed independent of the variations in the antibody content of the sera.

TABLE IV  
*Experiment 2*  
*Comparison of Groups on 44th Day*

Rabbit No.	Cutaneous reaction		Agglutinations							
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	Control
26-91	Group A Intracutaneously sensitized	++±	+	±	±	—	—	—	—	—
26-92		+±	++++	+++	+++	±	—	—	—	—
26-93		+±	+++	+	—	—	—	—	—	—
27-31		++++	+++	+++	++	+	—	—	—	—
26-95		+++	++±	++±	+++	+	±	±	—	—
26-96		+++	++	±	—	—	—	—	—	—
26-97		++++	++	++	+	—	—	—	—	—
26-98	Group B Intravenously immunized	±	++++	+++	+++	++±	+	±	—	—
26-99		++	++++	+++	++	+	±	—	—	—
27-00		±	++++	+++	+++	+	±	—	—	—
27-01		+	++++	++++	+++	++	+	±	—	—
27-02		±	+++	++	+	+	—	—	—	—
27-03		±	++++	++++	+++	++±	+	—	—	—
27-04		+	++±	++±	++±	++±	±	++	—	—
27-06	Group C Normal	±?	±	±	—	—	—	—	—	—
27-08		±?	—	—	—	—	—	—	—	—
27-09		±	—	—	—	—	—	—	—	—

# DISCUSSION

In spite of using antigens of such varying properties as vaccines prepared from virulent streptococci and living cultures of relatively avirulent green streptococci, the responses were in accord with one another and with previous observations. In the first experiment the total dose of antigen given in the form of vaccine over a period of 70 days was obtained from only 1.661 cc. of broth culture: in the second

a total 0.1349 cc. of living broth culture was administered within a period of 34 days. In spite of these relatively small inocula the animals, which had numerous areas of focal inflammation following intracutaneous inoculation, gave evidence of distinct hypersensitiveness. When, on the other hand, the route of preliminary inoculation was intravenous, and the animals gave no gross evidence of focal infection, the response to intracutaneous tests was much less than that of the intracutaneously inoculated groups, but slightly greater than that of normals. In a previous communication (3) it was noted that animals previously immunized with large doses of green streptococci responded to subsequent intracutaneous inoculation with lesions smaller than those of normal animals similarly tested, but this occurred only when the immunization was prolonged and intense; when, on the other hand, the immunization was short and mild such a diminution in response to inoculation was not so conspicuous. Some subsequent experiments have also indicated that the obtaining of a distinct state of hyporeactivity is dependent upon strong immunization. The results here presented, moreover, indicate that when the quantity of bacterial substance introduced intravenously is small and distributed over a long period, the resulting state of the tissues might possibly be designated as slightly hypersensitive when compared with that of normal animals; but it should again be noted that this degree of hypersensitiveness was much less than that of the animals which had suffered from multiple areas of focal inflammation. Another difference, not made evident by measurement alone, was constantly present: the lesions of the intravenously immunized animals were hard, nodular, and shotty on the 1st and 2nd days following intracutaneous inoculation, compared with small flat lesions in the normal animals and large edematous reactions in the cutaneously sensitized group. We regard the nodular type of response as indicative of the so called "hypoergic" immune state; and it is noteworthy that this qualitative type of reactivity was induced by such small intravenous inocula. It seems evident that only when the intravenous immunization is intense and prolonged is the quantitative "immune response" smaller than that of normals.

The evidence concerning the lack of quantitative relationship between agglutinin content of the blood serum and degree or type of

cutaneous response appears quite clear. Fortunately, the amount of bacterial substance given stimulated almost similar degrees of agglutinin formation in the two groups of animals receiving vaccines; and in the animals inoculated with living cultures of green streptococci the group injected intravenously had only slightly higher titer in the serum obtained on the 18th day than was present in the cutaneously sensitized group, but there was marked difference in the degree of hypersensitivity of the two groups. After an interval of 4 weeks, during which the animals received small inocula on only three occasions, the agglutinin titer of the serum from the intravenously immunized group was considerably enhanced, while that of the other group was only slightly raised; but as in previous experiments, no parallelism could be determined between the degree of hypersensitivity and the amount of agglutinin in the different animals. This, of course, does not prove a complete lack of relationship between antibody formation and hypersensitivity, because the antibody content of the tissues, in which the hyperergic reactions are made evident, is not necessarily reflected in the agglutinin content of the blood serum.

In human subjects Tuft (5) has recently observed fairly well marked cutaneous hypersensitiveness to be induced by intracutaneous injection of very small doses of horse serum; and Kohler and Heilman (6) demonstrated a slightly higher degree of cutaneous sensitization in children previously injected with small amounts of rabbit serum intracutaneously, than in those previously treated with similar doses intravenously. Such observations would make it appear that the cutaneous tissues respond in a similar manner to both intact bacteria and to soluble proteins. But the work of Opie (7) has demonstrated that with coagulable proteins the intensity of the Arthus phenomenon is roughly proportional to the antibody content of the serum against the antigen used for inducing the cutaneous reaction, a phenomenon not in accord with our findings with intact streptococci.

The size of the inocula approximated somewhat the amount of bacteria that may be active in human infections, or of vaccines used for treatment of patients. Comparative judgment of the relationship of dosage is difficult, however, because of the difference in size between rabbit and man. No attempt was made to increase the degree of immunity by doubling each successive dose; but rather an effort

was made to use the smallest dose for effective sensitization. These experiments, as well as others of a similar nature, indicate that the place in which the streptococci act in the animal body plays a very important rôle in the subsequent type of reactivity of the tissues, and that focal reactions are apparently more important factors in conditioning hypersensitiveness than is the antibody content of the blood serum.

#### SUMMARY

1. Rabbits were rendered very hypersensitive by relatively small doses of green streptococci given intracutaneously, and somewhat less hypersensitive by similar doses of heat-killed vaccine prepared from hemolytic streptococci.

2. Animals receiving the same doses intravenously gave, upon subsequent testing, lesions slightly more marked than normal controls; but these lesions were qualitatively hard and nodular compared with the large edematous lesions in the cutaneously sensitized group.

3. There was no parallelism between the degree of cutaneous or ophthalmic hypersensitivity and agglutinin titer in the blood serum.

4. Bacterial hypersensitivity to whole streptococci appears to depend more upon previously induced focal infection than upon circulating antibodies.

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## DETERMINATION OF LUNG VOLUME BY RESPIRATION OF OXYGEN WITHOUT FORCED BREATHING

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Since 1679 when Borelli (1) first endeavored to measure the air in the lungs interest has attached to the physiological and clinical significance of lung volume determinations. That portion of the pulmonary air which can be expired, either by usual or forced expirations, can be readily measured by spirometers. The residual air which remains in the lungs, however, requires other methods.<sup>1</sup>

Of these, two types have proven practical, the nitrogen dilution method and the hydrogen mixture method.

In the nitrogen dilution method, which has been reviewed by Lundsgaard and Van Slyke (2), the subject makes 5 or more forced respirations to and from a bag containing a measured volume of 2 or 3 liters of pure oxygen. Thereby the nitrogen of the pulmonary air is diluted with a known volume of oxygen. From the extent to which the nitrogen content of the gas mixture is diluted below the point, 79.1 per cent, found in ordinary pulmonary air, the volume of nitrogen, and hence of air, in the lungs at the beginning of the experiment can be calculated. Such an experiment must be completed in so short a time, that the difference between the volume of oxygen absorbed in the lungs and the volume of carbon dioxide excreted is not sufficient to affect significantly the total gas volume, and that the accumulation of carbon dioxide does not become great enough to make the gas mixture intolerable to breathe. To achieve mixture of the air in the lungs

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<sup>1</sup> For a discussion of the various fractions into which authors have divided the total lung volume the reader is referred to Lundsgaard and Van Slyke (2). The two main fractions are the "vital capacity," which is the volume of air that can be taken in by a maximal inspiration following a complete expiration, and the "residual air" left in the lungs after a complete expiration. The "middle capacity" is the volume of air held in the lungs at the middle of a normal respiration. For measurement of the "middle capacity" as well as the "residual air" the dilution method or some equivalent must be used.



with the oxygen in the bag in the permissible 20 or 30 seconds, it is necessary to accomplish the mixture in 5 or 6 respirations. These must approach in size the vital capacity of normal subjects, or mixture will not be complete. The simple dilution method is therefore limited in applicability to subjects who can cooperate, and who can make forced respirations of approximately normal extent.

To overcome these limitations Van Slyke and Binger (3) developed the hydrogen mixture method. The subject respire in a normal manner a mixture of oxygen and hydrogen, in which the volume of hydrogen is known, and the amount of oxygen need not be accurately measured, but is sufficient to maintain respiration for the 5- or 6-minute period found necessary for complete mixture of the gases when the respiration is of only ordinary depth. The carbon dioxide is removed by a scrubber. At the end of the period, oxygen and carbon dioxide in a sample of the respired gas are removed by absorption with alkaline pyrogallate, and the ratio  $N_2/H_2$  in the residual mixture of these two gases is determined by analysis. From this ratio and the known volume of  $H_2$  in the respired gases, the volume of  $N_2$  in the lungs is calculated as

$$\text{Vol. } N_2 = \text{Vol. } H_2 \times \frac{N_2}{H_2},$$

and the volume of air is estimated by dividing this volume of  $N_2$  by 0.791, the proportion of  $N_2$  present in the original pulmonary air.

The hydrogen respiration method can be carried out without cooperation of the subject, and has proved its practicality. Although Van Slyke and Binger used a spirometer, the method can be carried out with only a rubber bag attached to a soda-lime scrubber. The only drawbacks are those necessarily connected with the use of hydrogen for physiological experiments. Every lot of hydrogen must be tested for arsine, since some samples of commercial hydrogen contain this gas in sufficient amount to produce fatal intoxication. Furthermore, one is working with an explosive mixture of hydrogen and oxygen, ignition of which by flame or spark must be guarded against.

To avoid these drawbacks, we have in the present paper devised a technique which permits lung volume determinations without forced breathing, and requires respiration of no extraneous gas other than oxygen. The oxygen is respired from a spirometer, the carbon dioxide being continuously removed by a scrubber. At the end of the period the volume of gas in the spirometer is measured to ascertain the extent to which the pulmonary nitrogen has been diluted. The

calculation is then performed as in the simple nitrogen dilution method. The difference from the latter is that here the oxygen volume, with which the pulmonary air is diluted, is determined by measurement on the spirometer at the end of the respiratory period, instead of being taken as the oxygen volume measured at the beginning. Hence the respiratory period, as in the hydrogen method, can be prolonged as much as may be necessary to obtain mixture of the gases with ordinary respiration.

Since it is difficult to discuss the technique without referring to the factors involved in the calculation, the latter will be considered first.

### *Calculation*

The basic equation of the dilution method (2) is

$$(1) \quad V_L = V_{O_2} \times \frac{N_2}{79.1 - N_2}$$

$V_L$  = volume of air in lungs when the subject is connected to oxygen bag or spirometer;  $V_{O_2}$  = volume of oxygen, from bag or spirometer, with which the pulmonary air is diluted;  $N_2$  = per cent of nitrogen found in the respired gas mixture at the end of the period; 79.1 is the average per cent of nitrogen found in pulmonary air by Lundsgaard and Van Slyke (2).

When spirometers of the usual types are employed, the total volume of gas in the spirometer is the sum of the volume indicated on the scale plus the volume in the tubing, connections, scrubber, etc., which is not indicated by the scale. If we indicate the volume shown on the scale by  $V_S$  and that held in the dead space as  $V_D$ , and substitute their sum for  $V_{O_2}$  in Equation 1, we obtain:

$$(2) \quad V_L = (V_S + V_D) \frac{N_2}{79.1 - N_2}$$

The value of the dead space  $V_D$  is found in independent experiments with the spirometer used. In place of the lungs a bottle containing a known volume of atmospheric air is substituted and this air is mixed with oxygen in the spirometer. (Or oxygen is placed in the bottle and air in the spirometer.) By a rearrangement of Equation 2,  $V_D$  is then calculated, as will be detailed later.

### *Apparatus*

The familiar Roth-Benedict (4) and Krogh apparatuses for determination of basal metabolism can be used without alteration for lung volume by this method. The only disadvantage is the rather

large dead space. Because of it from 12 to 15 washings with oxygen are required before each determination to replace the air in the spirometer completely with oxygen.

To obtain an apparatus which has smaller dead space, and which can be constructed at small cost from ordinary laboratory equipment, one of the writers (S.) devised that shown in Fig. 1.

*Z* is a three-way stop-cock, one end of which is connected to the vacuum, the other to a high-pressure tank of pure oxygen. The rubber stopper *T* fits the rubber mouth piece *O*. The aluminum three-way stop-cock *S* of 20 mm. bore is made to communicate with the outside air, or through *O* with the apparatus. *I* and *E* are rubber spirometer valves (inlet and outlet), enclosed in glass jackets. *X* is a 500 to 750 cc. bottle with bottom removed, containing soda-lime. Copper

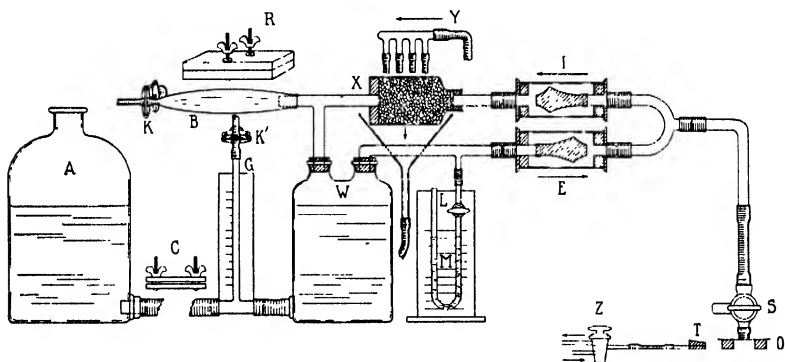


FIG. 1. Apparatus for determination of lung volume by the dilution method, without forced breathing.

gauze of not too fine mesh, or cotton loosely packed, may be used to keep the particles within the bottle. Running water enters the cooling system at *Y*, is distributed along the sides of the bottle, and runs off into a funnel. *B* is a rubber breathing bag, of 5 or 6 liters capacity. *R* is a press, something like that used for holding tennis racquets. It consists of two pieces of wood large enough in area to cover all but the ends of the bag *B*. *B* is also in communication with the Woulfe bottle *W* of 8 liters capacity, containing water. *G* is a tube with a millimeter scale. The bottle *W* is calibrated on the scale *G* by pouring in measured volumes of water, 300 to 400 cc. at a time, so that definite volumes of gas in *W* correspond to definite scale readings of the meniscus in *G*. The points are plotted on a curve, from which scale readings can be converted into liters of gas present in *W* (1 mm. on the scale of *G* corresponds to about 30 cc. volume in *W*). Reading *G* to 0.5 mm. in such an apparatus gives gas volumes in *W* to  $\pm 15$  cc. The flow of water through a suitable length of rubber tubing (of 32 mm. bore) between

*A* and *W* is controlled by the clamp *C*, placed as near as possible to the volume gage *G*. *M* is a water-filled manometer with a millimeter scale, connected with the rest of the system through the stop-cock *L*, which is of at least 2 mm. bore. All of the connecting tubes through which respired air passes are of heavy-walled glass or rubber tubing, of an internal diameter (23 mm.) sufficiently large to avoid resistance to respiration. In order to make the rubber tube connections at the bottom outlets of *A* and *W*, it may be necessary to seal a piece of glass tubing into each outlet. This may easily be done by the use of de Khotinsky cement. No temperature control is necessary, since the change in temperature of the gas contained within the system is so small during the period of a determination that it may be neglected. The zero point at the top of scale *G* is 3 or 4 cm. below the level of the stoppers in *W*.

#### *Determination of $V_D$ , the Dead Space of the Apparatus*

The apparatus is first filled with atmospheric air. If it has contained another gas, it is filled with air and emptied 12 to 15 times to replace other gases entirely with air of atmospheric composition. Then the air content is reduced to that of the dead space, and is mixed with a known volume of oxygen, as follows.

*Krogh or Roth Spirometer.*—The bell is pressed down until the indicator points to the zero mark on the scale so that no air except that in the dead space remains in the spirometer. Then the mouth piece of the spirometer is connected with a gas container holding a known volume of oxygen. An aspirator bottle like *A* in Fig. 1 serves as such a container. It is provided at the top with a stopper and a cock by which gas can be admitted and let out, and is connected at the bottom with a similar bottle which is filled with water. A mark is made on the stoppered bottle showing the level at which water stands in it when 5 liters of gas are present. The bottle thus calibrated is first filled to the stopper with water, and then pure oxygen is run in until the water has fallen to the 5-liter mark, the level of the water in the connected bottle being kept even with that in the calibrated one. The calibrated bottle is then connected with the spirometer by a short narrow tube bearing a perforated stopper which fits into the hole in the mouth piece of the spirometer. By raising and lowering the other bottle the oxygen is alternately forced into the spirometer and withdrawn from it 15 times, so that a uniform mixture of the oxygen with the air in the spirometer is obtained. A sample of the mixed gas is then analyzed for nitrogen.

In the above procedure, for economy's sake, oxygen is placed in the bottle at the start, and air in the spirometer, instead of *vice versa*.

The calculation of the dead space is similar to that of lung volume in Equation 1.

$$(3) \quad V_D = V_{O_2} \times \frac{N_2 - a}{79.04 - N_2}$$

In this case  $V_{O_2}$  represents the volume of oxygen measured into the bottle and then mixed with the air in the spirometer.  $V_{O_2}$  is 5 liters when the technique is carried through as above directed.  $N_2$  represents the per cent of  $N_2$  found in the gas mixture by analysis. The per cent of  $N_2$  present as impurity in the oxygen used is represented by  $a$ . The  $N_2$  content of atmospheric air is 79.04 per cent.

*Sendroy Apparatus.*—After the apparatus (Fig. 1) is washed out with air, the bag  $B$  is pressed in clamp  $R$  and the water in  $W$  is raised till the zero mark on  $G$  is reached, so that all the air except that in the dead space is removed from the apparatus. A bottle containing a measured volume of pure oxygen is attached to the mouth piece, clamp  $R$  is then removed from the bag, and the rest of the determination of  $V_D$  is carried out in the same manner as with the spirometers. In this case, however, the volume of oxygen used is 1 instead of 5 liters, and an aspirator bottle of 3 liters capacity is calibrated to hold the oxygen.

### *Lung Volume Determination*

*Preliminary Washing of Gases Other than Oxygen Out of Roth or Krogh Spirometer.*—Either of these instruments is filled as completely as possible with oxygen and emptied 12 to 15 times to remove all nitrogen. Washing of either of these spirometers requires about 90 liters of oxygen.

*Preliminary Washing of Sendroy Apparatus.*— $W$  is almost filled with water and clamp  $C$  is closed. Then the stopper  $T$  is inserted into the hole in the mouth piece. One outlet of the three-way cock  $Z$  connects with a suction pump and the other with an oxygen tank. Suction is applied through  $Z$  until the bag  $B$  is nearly deflated. Strong negative pressure is not applied because it might start leaks in the apparatus. By turning  $Z$ , enough oxygen to fill the gas bag is alternately admitted and withdrawn 10 times. The washing can

thus be completed in 2 or 3 minutes, and requires about 40 liters of oxygen.

*Addition of Oxygen for the Determination.*—With the dead space already filled with pure oxygen, enough more is run into the apparatus to fulfil the requirements of the subject for the duration of the experiment. For a resting subject 3 liters for 5 minutes and 6 liters for 10 provide more than enough.

With the Krogh or Roth apparatus one merely admits 3 or 6 liters of oxygen, measured by the rise of the pointer on the scale, for a 5- or a 10-minute period.

With the Sendroy apparatus the oxygen is admitted as follows. The bag *B* is flattened by clamping it with *R*. *T* is then inserted into the mouth piece *S*, and enough oxygen is wasted through cock *S* into the outer air to wash the air out of this cock. Then oxygen is admitted into the apparatus until the water level in *W* has fallen to a point indicating that the desired amount of gas has been admitted. (This level can be previously determined and indicated by a mark on bottle *W*.) A reading,  $V_{s_1}$ , is made on scale *G*. Clamp *R* is then removed from the bag and the latter is filled with gas from *W*. Clamp *C* is then closed, and bottle *A* is left elevated above *W*.

*The Respiration Period.*—The determination is carried out in the same way with any of the three types of apparatus.

The subject, with nostrils clamped by a nose piece, is connected to the apparatus by means of the mouth piece, and the cock *S* is so turned that room air is breathed for several normal respirations. The subject then brings his lungs to the desired position (for residual air he expires as completely as possible), and the cock is turned to connect him with the spirometer or bag. The subject then respire normally for the desired period. 5 minutes are sufficient to obtain a complete mixture for any normal subject. Longer periods may perhaps be needed when the respirations are very shallow. At the end of the period the subject brings his lungs to the same position as at the beginning and the cock *S* is turned off.

In the Sendroy apparatus it is necessary to admit additional gas into the bag from *W* at intervals during the period. This is done by opening clamp *C* and admitting water from *A* into *W*. So much must

not be admitted at any time that the bag is sufficiently filled to offer resistance to expiration. In particular, when residual air is to be determined, enough space must be left in the bag to receive the final maximal expiration.

*Final Gas Measurements and Sampling.*—In the Krogh or Roth spirometer the gas volume is read on the scale. The total gas volume in the extrapulmonary part of the system is then calculated by adding the volume,  $V_{S_1}$ , indicated by the scale, to the dead space,  $V_D$ , previously determined. A sample of gas from the spirometer is then drawn for gas analysis.

With the Sendroy apparatus the following procedure is used. The bag is compressed in the clamp  $R$  and the gas is thereby completely driven from the bag into  $W$ , as it was when the dead space was determined. The volume  $V_{S_1}$  is then read on the scale  $G$ . During the reading the cock of  $M$  is opened and the bottle  $A$  is set at such a level that the water menisci in the two limbs of manometer  $M$  are at the same level, indicating exact atmospheric pressure in the system.

To obtain a sample the bag is then refilled with gas from  $W$ , and the sample is withdrawn from outlet  $K$  of the bag.

*Analysis.*—The nitrogen in samples of the mixed gases is determined as the residual gas left after absorbing, with alkaline pyrogallol or hyposulfite solution, the oxygen and the small amounts of  $\text{CO}_2$  that may have escaped the scrubber. Any suitable gas burette serves for the measurements. A convenient procedure with use of the manometric apparatus of Van Slyke and Neill (5), instead of a gas burette, has been described by Van Slyke and Sendroy (6). It is also especially adapted to determination of the small amounts of nitrogen or hydrogen present as impurity in the oxygen.

### Calculation

If the oxygen gas used contains no significant amount of impurity in the form of  $\text{N}_2$  or other inert gas, determinable as  $\text{N}_2$  by the method of analysis used, the calculation is made by Equation 4.

$$(4) \quad V_L = (V_{S_1} + V_D) \frac{N_2}{79.1 - N_2}$$

If, however, the oxygen used contains amounts of nitrogen which significantly affect the result, the calculation must be made by Equation 5, in which allowance is made for such impurity.

$$(5) \quad V_L = \frac{V_D (N_2 - a) + V_{S_2} N_2 - V_{S_1} a}{79.1 - N_2}$$

In these equations the symbols,  $V_L$ ,  $V_D$ ,  $a$ , and  $N_2$  have the same significance as in Equations 1, 2, and 3.  $V_{S_1}$  represents the volume of gas read on the scale of the spirometer at the beginning of the determination,  $V_{S_2}$  the volume of gas read on the scale of the spirometer at the end of the period.

Equation 5 is derived as follows. Using the above symbols, and in addition  $V_{N_2}$  to express the total volume of nitrogen in the system of lungs + spirometer, we may calculate the values of  $V_{N_2}$  at the beginning and end of the respiratory period, respectively, by means of Equations 6 and 7.

$$(6) \quad V_{N_2} = 0.791 V_L + 0.01 a (V_{S_1} + V_D)$$

$$(7) \quad V_{N_2} = 0.01 N_2 (V_L + V_{S_2} + V_D)$$

Since the nitrogen volume,  $V_{N_2}$ , present in the system is the same at the beginning as at the end of the period, we may equate the right hand members of Equations 6 and 7. Doing so, and solving the resulting equation for  $V_L$ , we obtain Equation 5. If  $a = 0$ , Equation 5 becomes Equation 4.

From the lung volume calculated by either Equation 4 or 5 a small correction is to be deducted for the dead space in the mouth piece used to connect the subject with the spirometer. This dead space is that contained in the tube between the mouth of the subject and the three-way valve, and is usually less than 100 cc. If the space is cylindrical it may be estimated as  $0.8 D^2 H$  cc., where  $D$  indicates the diameter of the cylindrical section of tubing involved and  $H$  is the length of this section, from the mouth end of the tube to the valve. Or this space may be measured simply by closing the valve on the side towards the mouth piece, and ascertaining the volume of water which must be poured in, in order to fill the space.



## EXPERIMENTAL

Determinations of lung volumes in the same normal subjects have been performed by the present method, and by the two others discussed in the introduction.

*The Dilution Method with Forced Breathing.*—The technique of Lundsgaard and Van Slyke (2) was followed. The subject breathed 5 or 6 times in and out of a rubber bag previously washed and filled 3 or 4 times with pure oxygen. The results were calculated according to Equation 1, and are given in Table I.

TABLE I

*Determination of Lung Residual Air by the Dilution Method with Forced Breathing*

Subject	Residual air
	<i>liters</i>
A	$V_L = 1.42$
	1.54
	1.42
	1.50
B	$V_L = 2.29$
	2.10
	2.34
	2.24

*The Present Dilution Method without Forced Breathing.*—A preliminary experiment was performed to determine the number of washings necessary with oxygen when the dead space in the Benedict-Roth spirometer was initially filled with air. With a dead space of about 5.9 liters, using about 5 liters for each washing, on the assumption that there is complete mixture of all the gases with each washing, one can calculate the number of washings needed to fill the system with pure oxygen. The result of such a calculation is given in Table II, Column 2. The next column indicates the results actually obtained by analysis after washing with tank  $O_2$  from the breathing end of the apparatus. The oxygen in the tank had been analyzed and found to contain 0.43 per cent  $N_2$ . Table II shows that washing is much more efficient than it would be if uniform mixture of each por-

TABLE II

*Calculated and Observed Results of Washing Benedict-Roth Spirometer Apparatus with Oxygen Containing 0.43 Per Cent N<sub>2</sub>*

No. of washings	Per cent N <sub>2</sub>	
	Calculated	Observed
Beginning	79.05	79.05
1	43.1	8.84
2	43.6	3.75
3	13.0	2.41
4	6.7	2.26
5	3.8	1.31
8	1.0	1.18
12	0.6	0.75
15	0.6	0.8

TABLE III

*Determination of Lung Residual Air by the Dilution Method without Forced Breathing. Roth-Benedict Spirometer Used*

$$V_D = 5.79 \text{ Liters}$$

Respiration time	Subject A	Subject B
<i>min.</i>	<i>liters</i>	<i>liters</i>
1	1.34	1.54
2	1.24 1.37	2.15
3	1.41 1.42	2.06 2.08
4	1.42 1.46	2.05 2.09 1.97
5	1.41	2.17 2.00 2.07
7.5	1.55	2.09
10	1.58	2.26

tion of oxygen occurred with the gas in the dead space, and that 12 to 15 washings suffice to clear the Roth-Benedict spirometer system of almost all nitrogen except that which is introduced with the tank oxygen. Apparently, when oxygen is run into the spirometer it does not rapidly mix with the gas there, but tends to form a layer by itself and to push the previous gas out.

TABLE IV

*Determination of Lung Residual Air by the Dilution Method without Forced Breathing.  
Authors' Apparatus Used*

$$V_D = 0.89 \text{ Liter}$$

Respiration time	Subject A	Subject B
<i>min.</i>	<i>liters</i>	<i>liters</i>
2		1.80
3	1.45	1.85
4	1.45	2.00 2.04
5	1.50	1.99 2.14 1.93
6	1.45	1.95 2.06
7	1.50	2.04
8	1.47	2.08 1.96

In subsequent determinations of  $V_L$  with the Benedict-Roth spirometer, the system was washed 15 times with 5-liter portions of oxygen, and the value of 0.75 per cent  $N_2$  was used for  $a$  in Equations 3 and 5. Four consecutive determinations of  $V_D$  for this system gave values of 5.74, 5.76, 5.83, and 5.78 liters. Table III indicates the results obtained for residual air.

In using the apparatus shown in Fig. 1, it was found that 10 washings of 4 liters each, with the same oxygen used before, reduced the

nitrogen content of the system to 0.54 per cent. This value was used in Equations 3 and 5, for the calculation of  $V_L$  determinations by this apparatus. The  $V_D$  values obtained were 0.87, 0.89, and 0.89 liter. The  $V_L$  values for the same subjects used in Table III are given in Table IV.

### *The Hydrogen Method without Forced Breathing*

The apparatus of Fig. 1 was tested to determine the practicability of its use in connection with the hydrogen method of Van Slyke and Binger. This has the advantage that no  $V_D$  determinations are necessary. It was thought that error might arise from loss of hydrogen by diffusion out of the rubber bag, hydrogen, because of its small molecules, being a rapidly diffusing gas. However, actual measurement showed that within the limit of error in reading the scale  $G$ , there was no loss of gas over a period of 30 minutes, when the bag and the system were filled with hydrogen.

Three determinations of  $V_L$  (residual air) for Subject A were performed. The hydrogen contents of the mixed gases were determined by the combustion method of Van Slyke and Hanke (7). The results, calculated as by Van Slyke and Binger (3), were 1.56, 1.58, and 1.62 liters for 4, 5, and 6 minutes respiration, respectively.

### *Discussion of Results*

The results outlined above indicate good agreement between all of the methods used. Apparently, within the analytical and physiological limits of error, both Subjects A and B were in equilibrium with either the Benedict-Roth or the new apparatus, during the respiration time of 3 to 7 minutes. Subject A was quite remarkably constant over a period of several minutes, by any one of the methods used. That similar results would be obtained with other subjects in all cases cannot be stated. Van Slyke and Binger found that one cannot generalize as to the length of time required to reach equilibrium, since several factors, including the initial volume of oxygen in the spirometer and the depth and rapidity of respirations, are of influence. These authors found, however, that with the hydrogen mixture method

equilibrium was obtained in a few minutes even with decompensated cardiac patients, and the conditions of the present method are so similar that the results of Van Slyke and Binger with regard to this point can apparently be applied.

Compared with the hydrogen mixture method (3) for determination of lung volume without forced breathing, the present procedure has the disadvantage of requiring a spirometer, which must be calibrated and have its dead space determined, while the hydrogen method requires only a rubber bag and a scrubber. However, the dead space can be measured with such accuracy that it does not significantly diminish the precision of the present method. The latter has, over the hydrogen procedure, the advantages that it obviates the necessity for the precautions required for the safe handling of hydrogen, requires the use of only one pure gas (oxygen), and simplifies the final analysis to the determination of only nitrogen, instead of nitrogen and hydrogen. For the subject, one method is as convenient as the other. The choice between them will depend upon the spirometer equipment available, and upon the facilities for handling and analyzing gases.

#### SUMMARY

A method is described for estimating the volume of air in the lungs by the familiar principle of mixing this air with a measured volume of oxygen, and determining the extent to which the nitrogen of the pulmonary air is diluted. By employing a scrubber to remove carbon dioxide, and by measuring the volume of gas in the extrapulmonary part of the system at the end instead of the beginning of the respiratory period, it is possible to prolong the period to as many minutes as are necessary for complete mixture of the gases, and thereby to carry out the estimation without forced breathing.

The determination can be carried out with the Roth-Benedict or Krogh spirometer, or, more economically, with the simple spirometer, shown in Fig. 1, assembled from ordinary laboratory equipment.

The method gives the same results as the hydrogen method of Van Slyke and Binger (3), and obviates the use and analysis of hydrogen. The relative advantages of the two methods are discussed above.

Dr. Ronald V. Christie has informed us that he has encountered considerable differences in the  $N_2$  content of the pulmonary air of different individuals. A gain in accuracy would therefore be made if this value were determined for each subject, and substituted for 79.1 in the calculation formulae.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

### I. THE DETERMINATION, BY SIMPLE ABSORPTION, OF CARBON DIOXIDE, OXYGEN, AND NITROGEN IN MIXTURES OF THESE GASES

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#### APPLICABILITY OF MANOMETRIC APPARATUS TO ANALYSIS OF GAS MIXTURES

In the original description of the manometric blood gas apparatus by Van Slyke and Neill (1924) it was pointed out that the apparatus could be used for air analyses. Details for adaptation of the apparatus to the different gas mixtures encountered in biological analyses were not, however, perfected at that time. Such details are now available.

The constituents of a gas mixture can be successively removed by absorption or combustion, and the amounts so removed estimated by the decrease in gas pressure registered on the manometer.

The absorption, in the cases of  $\text{CO}_2$  and  $\text{O}_2$ , can be carried out by admitting the necessary solutions to the chamber itself, so that transfer of the gas to other vessels for absorption is avoided. Such transfer can, however, readily be accomplished to modified Hempel pipettes when desirable.

Another procedure, especially adapted to estimation of a gas forming only a minute proportion of a mixture, is based on isolation of such a gas by absorbing it in the chamber of the apparatus with suitable reagents, such as alkali for  $\text{CO}_2$ , or reduced blood for CO. The unabsorbed gases are then ejected, and the absorbed gas is set free from solution and measured, as in blood gas determinations.

Analysis by combustion is carried out as easily as with ordinary volumetric apparatus. The manometric apparatus has an ad-



vantage in analysis of highly explosive mixtures, which in other methods are ordinarily diluted with inert gas to reduce their explosibility. Here such dilution and its resultant loss of accuracy are unnecessary, because the explosibility can be decreased merely by putting the mixture under reduced pressure during ignition.

In convenience, analysis of gas mixtures by the manometric apparatus is not inferior to methods of equal accuracy by the usual procedures with gas burettes and absorption bulbs. When the absorbing solution can be admitted into the chamber of the manometric apparatus, making gas transfer unnecessary, the manometric procedure is likely to be the more convenient.

In the maximum accuracy attainable, when simple absorption procedures are used to remove the gases determined, the manometric technique is inferior to the more refined types of volumetric methods. A 0.1 mm. division on the 500 mm. length of manometer ordinarily employed corresponds to 0.0002 of the gas measured, and marks the limit of manometric accuracy in this type of analysis. Volumetric gas burettes, on the other hand, for special purposes can be made with relatively large volumes and with small bores at the points where measurements are made, so that a 0.1 mm. division corresponds to 0.0001 of the gas measured, as in the ordinary 10 cc. Haldane apparatus, or to a still smaller fraction if it is desired. For many purposes, however, the accuracy of 0.0002 suffices, and the convenience of the manometric procedure then may make it the one of choice.

Furthermore, the manometric technique is not limited to simple absorption procedures. The isolation method with the manometric apparatus makes possible a great extension of its refinement, and permits determination, without unusual precautions, of minute proportions of gases, with an accuracy such as can be attained with volumetric methods only by extreme precautions and use of special apparatus. Thus  $\text{CO}_2$  in atmospheric air can easily be determined manometrically by the isolation method with a precision approaching 0.0003 of a volume per cent of the air, and small amounts of carbon monoxide to 0.001 volume per cent.

The manometric method also has an advantage for determining small amounts of inert gas admixed with great amounts of other gases capable of removal by absorption. In such a case the sample can be measured by its pressure at 50 cc. volume, and the

residual unabsorbed gas by its pressure at 0.5 cc. volume, so that its pressure is multiplied 100-fold. Thus 1 volume per cent of nitrogen present as impurity in oxygen can be determined with an accuracy approaching 0.001 volume per cent.

In all the methods outlined in the present series of papers, the manometric apparatus described by Van Slyke and Neill (1924) and Van Slyke (1926-27) is used without alteration. The chamber could be modified, so that readings could be taken with the gas at other volumes than the 0.5, 2, and 50 cc. provided by the original chamber, but such modifications have as yet not been found necessary. The same apparatus, used for determination of the blood gases and various other substances by methods previously published in this *Journal*, can also be used without alteration for the gas analyses described in this and the succeeding papers.

Methods A and B described in this paper for determination of  $O_2$ ,  $CO_2$ , and  $N_2$  in air by simple absorption are capable of being carried out with errors not exceeding 0.1 volume per cent, and usually within 0.05 volume per cent. When greater accuracy is required, the isolation method is used for  $CO_2$  (Van Slyke, Sendroy, and Liu, 1932) and the combustion method for  $O_2$  (Van Slyke and Hanke, 1932), as described in accompanying papers of this series. Method C, described in this paper for small amounts of  $N_2$  or other inert gas present in oxygen or carbon dioxide, is of the highly accurate type mentioned above.

#### GENERAL POINTS OF TECHNIQUE FOR APPLICATION OF MANOMETRIC APPARATUS TO ANALYSES OF GAS MIXTURES

*Extension of Manometer Scale for Low  $p_0$  Readings*—The zero readings with the mercury in the chamber at the 50 cc. mark fall in the low part of the manometer tube opposite the bottom of the chamber. Most of the closed tube manometers made for the Van Slyke-Neill apparatus before 1931 have scales which fail by 10 or 20 mm. to extend so low, since none of the methods used prior to this time involved zero readings with more than 2 cc. of gas space in the chamber. It is, however, not difficult to improvise an extension of the scale to make possible zero readings at the lower point. On transparent paper a ladder of parallel lines 1 mm. apart is made with black India ink. Each line is made long enough to extend half way around the manometer tube, except that

every fifth line is made a little longer to facilitate counting the distances. The strip of paper with the lines is cut of sufficient width to extend two-thirds of the way around the manometer tube. It is then pasted onto the tube in such a way that the open third is towards the observer's eye, and the uppermost mark on the paper covers the zero mark on the glass scale. If such a scale extension is used, a convenient way to employ it in connection with the scale already present is to add 100 mm. to each reading made on the latter, and consider the zero point to be the point on the extrapolated scale 100 mm. lower than the original zero of the glass scale.

### *Temperature Control and Corrections*

*Control*—In so far as atmospheric conditions are concerned, the manometric apparatus with the closed manometer is independent of changes in barometric pressure, because the manometer and chamber of the apparatus form a closed system.

Temperature changes, however, occurring in the manometric apparatus in the course of an analysis, cannot be automatically compensated by a device like the thermobarometer. The manometric apparatus is therefore used under conditions to minimize temperature changes during the short time required for an analysis. If the temperature registered by the thermometer in the water jacket of the chamber of the apparatus differs from that registered by a thermometer in the air of the room at the same level, the water jacket is warmed or cooled by wrapping it for a minute or longer in a towel wet with hot or cold water. The chamber is then shaken and the temperature on its thermometer noted. It is desirable to bring it within 0.2° or 0.3° of the room temperature. During an analysis the room temperature is kept as constant as possible.

*Corrections*—If significant temperature changes do occur during an analysis, corrections for them must be applied. The corrections are estimated from the following considerations.

The procedure in most of the analyses is to measure the sample by its pressure,  $P_s$ , as described below for "Measurement of sample," then to remove the gas determined, and finally to measure the pressure,  $P_R$ , of the residual gas. The constituent gas determined is measured by the difference,  $P_s - P_R$ .

$P_S$  of the sample is measured as the difference between two manometer readings,  $p_0$  taken with the manometer chamber empty, and  $p_1$  taken after admission of the sample. These two readings are taken within such a short time interval that there is ordinarily no significant temperature change between them. Similarly  $P_R$  is measured as the difference between two quickly succeeding manometer readings, one before and one after the residual gases have been ejected from the chamber.

Between the observations of  $P_S$  and  $P_R$ , however, a period of several minutes may elapse during which the constituent gas determined is in process of removal by absorption or combustion, so that temperature changes of  $0.2^\circ$  or  $0.3^\circ$  may occur between the  $P_S$  and  $P_R$  measurements. For comparison with  $P_S$  in the calculation of results,  $P_R$  must in such a case be corrected, in order to obtain the value it would have at the temperature of the sample measurement. For this purpose the observed value of  $P_R$  is multiplied by the factor,  $\frac{T_S}{T_R}$ , where  $T_S$  represents the absolute temperature (centigrade  $+273^\circ$ ) observed in the water jacket of the chamber at the time of the sample measurement, and  $T_R$  is the absolute temperature of the final  $P_R$  measurement. The correction amounts to about 1 part per 3000 for  $0.1^\circ$  temperature change.

$$(1) \quad P_R \text{ corrected to } T_S = P_R \text{ observed} \times \frac{T_S}{T_R}$$

The following values of the factor  $\frac{T_S}{T_R}$  are used for the conditions encountered and are convenient to have at hand. When  $T_S$  is less than  $T_R$ , the factor  $\frac{T_S}{T_R}$  has the value 0.9997 for  $0.1^\circ$  difference between  $T_S$  and  $T_R$ , 0.9993 for  $0.2^\circ$ , 0.9990 for  $0.3^\circ$ , and 0.9986 for  $0.4^\circ$ . When  $T_S$  exceeds  $T_R$  the values of the factor are 1.0003 for  $0.1^\circ$  difference, 1.0007 for  $0.2^\circ$ , 1.0010 for  $0.3^\circ$ , and 1.0014 for  $0.4^\circ$ .

In the analyses described in this paper,  $P_R$  is represented by the pressure,  $P_{N_2}$ , of the residual nitrogen left after absorption of the  $CO_2$  and  $O_2$ .

The above correction is the only one that need ordinarily concern the analyst who uses the methods described in this series of papers. The precautions outlined under "Control" are usually sufficient, as stated above, to prevent significant temperature changes in the interval between the two successive manometer readings involved in the measurement of any one gas portion.

### *Technique of Manometer Readings*

Before every manometer reading in these gas analyses, the mercury meniscus in the chamber is lowered below either the 2 or the 50 cc. mark, and is then brought slowly up to the mark by admitting mercury from the leveling bulb while the latter rests level with the bottom of the chamber. While the mercury in the chamber is rising to the mark it is observed with a magnifying glass (a good reading glass serves well), and the cock from the leveling bulb is closed just as the top of the curved mercury meniscus reaches the mark. With practice one can bring the mercury in the chamber thus to the same level with a constancy of 0.1 mm. It is essential that the mercury surface should always be brought to the mark in the same manner from below upwards, and never from above downwards, because the manometer readings obtained after these two different approaches differ, slightly but measurably. After the mercury is at the mark in the chamber the reading on the manometer is taken. If there is any doubt concerning the accuracy of the placing of the mercury meniscus in the chamber, the mercury is lowered and brought up to the mark again, and the reading is repeated. In such duplicate observations the readings on the manometer (also with the help of a lens) should differ by not more than 0.1 mm.

### *Preparation of Manometric Chamber to Receive Gas Sample*

The manometric chamber is washed, if necessary, to free it from interfering solutions used in preceding analyses. Then all the water present is removed except the invisible film adherent to the walls. To remove excess water the mercury in the chamber is lowered to the bottom, and then is permitted to rise *slowly* to the top. The water which collects on the surface is expelled, and about 2 cc. of mercury are driven up into the cup above the chamber. The chamber is then evacuated and the mercury lowered to

the 50 cc. mark. If more water then collects on the mercury surface than will form a slight ring about the edge, the above procedure is repeated to finish the removal of the water. The repetition will not be necessary unless during the first removal the mercury was allowed at some point to rise too rapidly to permit the water to detach itself completely from the glass walls ahead of the ascending mercury.

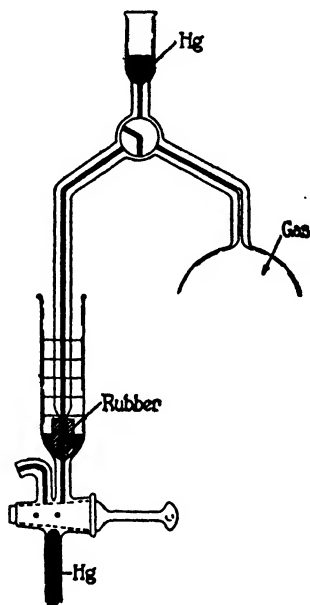


FIG. 1. Admission of gas sample into manometric chamber

The *zero reading*,  $p_0$ , of the apparatus, with the chamber free of gas and visible water, is now taken, as described above under "Technique of manometer readings," with the mercury meniscus at either the 2 or the 50 cc. mark, whichever is to be used in the analysis.

The tip of the capillary from the sample container is inserted into mercury in the cup of the chamber, as shown in Fig. 1. Any satisfactory container with a 3-way cock may be used, such as the familiar Barcroft tube, or the modified Hempel pipette of Van Slyke and Hiller (1928). The connecting capillary should be

narrowed at the tip, which is fitted with a rubber ring. Such a ring is made by cutting a section about 10 mm. long from a soft rubber tube of about 1 mm. bore with walls 2 mm. thick. The bottom of the ring is beveled slightly by grinding on an emery wheel or with sandpaper, so that it will fit the curvature of the bottom of the cup when pressed into the latter as shown in Fig. 1.

The manometric leveling bulb is first placed in the ring above the chamber, and mercury is forced up through the connecting capillary and 3-way cock of the sample container, displacing the air in its capillary, as shown in Fig. 1. During this operation the capillary is pressed into the bottom of the cup with one hand, so that the rubber ring makes a tight seal under the mercury, while the cocks are turned with the other hand.

#### *Admission of Sample Regulated by Volume*

For most gas analyses one takes a sample sufficient to give 450 to 550 mm. of pressure at either 2 or 50 cc. volume, according to whether a micro or macro analysis is to be made. Such a sample is of about 1.5 or 35 cc. volume at atmospheric pressure. Its admission can be regulated with sufficient accuracy as a rule by the following procedure.

The gas container and manometric chamber are arranged as shown in Fig. 1, and the leveling bulb of the chamber is placed about level with the bottom of the chamber. The cock connecting leveling bulb and chamber is left open, so that the contents of the chamber are under slight negative pressure. Gas from the sample container is then admitted to the chamber until the volume in the latter is approximately 1.5 or 35 cc. The volume can, with slight practice, be estimated with sufficient accuracy from the height of the mercury surface in the chamber above the 2 or 50 cc. mark.

Another procedure, which can be used also for samples of size not easily estimated from the marks on the chamber, is to admit the gas sample first to a container which holds at atmospheric pressure approximately the desired amount. From the container the sample is transferred to the manometric chamber by the technique shown in Fig. 9 of Van Slyke and Neill (1924). The water there shown in the container is here replaced by gas.

*Admission of Sample Regulated by Pressure*

For admission of samples intermediate between the 1 to 1.5 and the 30 to 35 cc. sizes the following procedure can be used. It requires somewhat more skill than admission regulated by volume, but has the advantage that it obviates the use of a special container for preliminary approximate measurement of the sample.

The apparatus is prepared and the  $p_0$  reading taken as described above. The sample container is then permitted to hang in position, with its cock closed and the tip of its capillary outlet immersed in the mercury of the cup of the chamber. The capillary of the gas container is filled with mercury as in Fig. 1. The mercury in the chamber is then lowered till it is about 10 mm. above the 50 cc. mark. The cock between leveling bulb and chamber is then closed, and the leveling bulb is rested in the medium position, level with the bottom of the evacuated chamber. The tip of the capillary from the sample container is then pressed into the bottom of the cup, as shown in Fig. 1, and the cock of the container is turned to connect container and chamber. Then the cock at the top of the chamber is slightly opened, just long enough to admit enough gas to depress the mercury level in the chamber a little below the 50 cc. mark. The mercury in the manometer rises 20 to 40 mm. The cock leading to the manometric leveling bulb is now opened to admit enough mercury to the chamber to make the meniscus rise again about 10 mm. above the 50 cc. mark; then enough gas is again let in to force the mercury a little below the mark. This is repeated one, two, or three times, until the mercury in the manometer has risen to about the desired final level. During this procedure one holds the capillary tube of the sample container in position with the left hand, while with the right hand one alternately opens the cock at the top of the chamber to admit gas, then the cock from the leveling bulb to admit mercury. The procedure is in fact simple, and the admission of the entire sample by alternate turns of the two cocks requires less than a minute. Before the last admission of gas, the amount of mercury admitted is regulated from previous experience so that the subsequent admission of enough gas to press the mercury down to the mark will raise the mercury column in the manometer to the desired height; e.g., the amount of mercury admitted before the last portion of



gas may need to be only enough to raise the meniscus 5 mm., instead of 10 or 15, above the 50 cc. mark in the chamber.

### *Measurement of Sample*

After the sample has been admitted by either of the above procedures, the sample container is removed and the bore of the cock at the top of the chamber is filled with mercury from the cup (this cock is gas-tight only when *both bores are filled with mercury*). The mercury meniscus is then lowered below either the 2 cc. or the 50 cc. mark, and is brought back to the mark as above directed. The manometer reading  $p_1$  is then taken, and the temperature of the chamber is read on the thermometer in the water jacket. The size of the gas sample is indicated by the pressure  $P_s$ , which the gas exerts at the chosen volume, either 2 or 50 cc.

$$(2) \quad P_s = p_1 - p_0$$

The *volume*,  $V_s$ , of the gas sample, reduced to 0°, 760 mm., can be calculated from  $P_s$  by the formula:

$$(3) \quad V_s = a \times \frac{P_s}{760} \times \frac{1}{1 + 0.00384t}$$

Here  $a$  is the volume (50 or 2 cc., as a rule) of the gas sample when its pressure,  $P_s$ , is measured, and  $t$  is the temperature centigrade. The formula is similar to the familiar one

$$V_{0^\circ, 760} = V_{t^\circ, B-W \text{ mm.}} \times \frac{B - W}{760 (1 + 0.00367t)}$$

In Equation 3,  $a$  represents the observed volume at which the gas is measured under  $P_s$ , instead of  $B - W$ , mm. of pressure. The coefficient 0.00384 for  $t$ , instead of the usual 0.00367, is employed in order to correct for expansion of mercury in the manometer with temperature, as explained on p. 540 of Van Slyke and Neill's paper (1924).

In calculating the results of analyses described in this paper only pressure figures are used, so that transformation of the sample measurement into terms of volume is not necessary. Equation 3 is given, however, as it is sometimes desirable to measure a gas

by pressure in the apparatus, and then to calculate the volume under standard conditions for other purposes.

#### METHOD A. DETERMINATION OF OXYGEN AND CARBON DIOXIDE IN AIR

The gas sample is measured by the pressure it exerts at 50 cc. volume, and the diminutions of pressure noted after successive absorption of  $\text{CO}_2$  in the chamber with sodium hydroxide, and of oxygen with hyposulfite, are used to calculate these gases. For  $\text{CO}_2$ , when it forms less than 10 per cent of the gas analyzed, this method is less accurate than the isolation procedure described in the second paper of this series (Van Slyke, Sendroy, and Liu, 1932), and for  $\text{O}_2$  the precision is less than in the method based on combustion with  $\text{H}_2$  given in Paper IV (Van Slyke and Hanke, 1932). The determinations by simple absorption are, however, most convenient and rapid, and are reliable within limits which are sufficiently precise for many purposes.

Gas samples are taken of 30 to 35 cc. volume, giving a pressure at 50 cc. of about 500 mm. of mercury. The manometer readings are reproducible to 0.2 mm., corresponding to 0.04 volume per cent of the sample. If errors of this size, but opposite in direction, were made before and after absorption of either gas, the resultant error in the analysis would be the sum, or 0.08 per cent of an atmosphere. However, by performing check readings for each observation of the manometer, it is possible to keep the usual error within  $\pm 0.05$  volume per cent (see Table I).

#### *Reagents*

*1 N Sodium Hydroxide*—The solution need not be freed of air for this analysis.

*30 Per Cent Sodium Hyposulfite in 2 N Potassium Hydroxide*—15 gm. of the hyposulfite,  $\text{Na}_2\text{S}_2\text{O}_4$ , are stirred up with 50 cc. of 2 N potassium hydroxide solution and filtered quickly through cotton into a 100 cc. flask, containing enough paraffin oil to make a layer 1 or 2 cm. thick.

Fieser's anthrahydroquinone- $\beta$ -sulfonate catalyst, which has proved its convenience in blood oxygen determinations (Van Slyke, 1927), is here left out of the hyposulfite solution, because the intensely red color of the catalyst would prevent reading the

mercury meniscus when the solution is in the chamber. The analysis could be accomplished in 1 minute less time with the sulfonate catalyst present, but it appears usually to be preferable to use the water-clear solution of hyposulfite without the catalyst.

For Methods A and B the solution is used without freeing it of dissolved air, because the oxygen is absorbed with the system at nearly atmospheric pressure. For Method C, however, the solution is freed of air as described on p. 535 of Van Slyke and Neill (1924). The air-free solution is kept over mercury and out of contact with air. A layer of oil would not protect it from absorption of sufficient atmospheric nitrogen to affect the results of analyses by Method C.

#### *Measurement of Sample*

The sample of 30 to 35 cc. is admitted into the apparatus as described above on p. 474, and  $P_S$  is measured as described on p. 476 for "Measurement of sample."

$$P_S = p_1 - p_0$$

#### *Absorption of CO<sub>2</sub>*

1 cc. of 1 N sodium hydroxide is measured accurately into the chamber from a stop-cock pipette in the manner shown in Fig. 3, p. 125, of a previous paper (Van Slyke, 1927). During the admission of the alkali the manometric leveling bulb is at the medium level, with the mercury surface in the bulb at about the height of the 50 cc. mark on the chamber. The cock between leveling bulb and chamber is left open, so that the contents of the chamber are under slightly negative pressure, and the chamber is about one-third full of mercury. After the alkali is admitted the chamber is shaken for 2 minutes so that the alkali will absorb the CO<sub>2</sub> from the gas sample. On account of the weight of mercury in the chamber the shaking is somewhat slower than in most analyses with this apparatus. During the shaking, the cock between the chamber and its leveling bulb is left open. The 10 or 15 cc. of mercury in the chamber and the alkali solution over the mercury are thrown about in such a way that thorough contact between gas and liquid is obtained, and CO<sub>2</sub> absorption is completed in 2 minutes. It is usually, in fact, finished during the 1st minute.

After absorption is finished the manometer reading  $p_2$  is taken with the meniscus of the *mercury* (not of the water solution) at the 50 cc. mark in the chamber.

### *Absorption of Oxygen with Hyposulfite*

After the  $p_2$  reading has been taken the cock between the leveling bulb and the chamber is opened and 3.00 cc. of hyposulfite solution, accurately measured from a calibrated stop-cock pipette, are run into the chamber in the same manner in which the 1 cc. of sodium hydroxide was added. The absorption of oxygen is accomplished by shaking the chamber in the same manner as for  $\text{CO}_2$  absorption, except that for oxygen 3 minutes instead of 2 are taken.

After the absorption is completed the meniscus of the *mercury* is again brought to the 50 cc. mark and  $p_3$  is read.

The gas is then ejected from the top of the chamber without loss of any of the solution (Van Slyke, 1926-27, p. 240) and  $p_4$  is read with the 4 cc. of solution, but with no gas, in the chamber, and with the mercury meniscus again at the 50 cc. mark. The analysis is now complete.

### *Calculation*

All quantities of gas measured are calculated in terms of the pressure exerted with the gas at 50 cc. volume and at the temperature of the  $P_S$  measurement. The sample is calculated as:

$$P_S = p_1 - p_0$$

The pressure of  $\text{O}_2 + \text{N}_2$  is measured at 49 cc. volume. To calculate it for 50 cc. volume, therefore, it is necessary to multiply the observed pressure by  $\frac{49}{50}$ , or 0.98.

$$(4) \quad P_{\text{O}_2 + \text{N}_2} = 0.98 (p_2 - [p_0 + c])$$

The significance of the  $c$  correction will be discussed below.

The pressure of the  $\text{CO}_2$  is calculated by subtracting the pressure of the  $\text{O}_2 + \text{N}_2$  from that of the total sample.

$$(5) \quad P_{\text{CO}_2} = P_S - P_{\text{O}_2 + \text{N}_2}$$

The pressure of the N<sub>2</sub> at 46 cc. volume is measured as  $p_3 - p_4$ . At 50 cc. therefore one calculates:

$$(6) \quad P_{N_2} = 0.92 (p_3 - p_4)$$

The oxygen is calculated by subtracting the N<sub>2</sub> from the O<sub>2</sub> + N<sub>2</sub>.

$$(7) \quad P_{O_2} = P_{O_2 + N_2} - P_{N_2}$$

The final results are calculated from the above data as follows:

$$(8) \quad \text{Per cent CO}_2 = \frac{100 P_{CO_2}}{P_S}$$

$$(9) \quad \text{Per cent O}_2 = \frac{100 P_{O_2}}{P_S}$$

$$(10) \quad \text{Per cent N}_2 = \frac{100 P_{N_2}}{P_S}$$

### *Remarks on Calculation*

As shown above, one must multiply the observed O<sub>2</sub> + N<sub>2</sub> and N<sub>2</sub> pressures by 0.98 and 0.92, respectively, in order to calculate the pressures at 50 cc. volume from those at 49 and 46 cc. In practice, the simplest way to make this calculation is to subtract 0.02 and 0.08 of their values from the observed pressures,  $p_2 - [p_0 + c]$ , and  $p_3 - p_4$ .

*Example of Calculation*—The data are from an analysis of laboratory air.  $c = 1.4$  mm. for the chamber used.

$p_1 = 551.4$	$p_2 = 561.8$
$p_0 = \underline{88.0}$	$p_0 + c = \underline{89.4}$
$P_S = 463.4$	$P_{O_2 + N_2} \text{ at } 49 \text{ cc.} = 472.4$
	$0.02 \text{ of same} = \underline{9.4}$
	$P_{O_2 + N_2} \text{ at } 50 \text{ cc.} = 463.0$

$$p_1 = \overset{\text{mm.}}{487.0}$$

$$p_4 = \underline{89.0}$$

$$P_{N_2} \text{ at 46 cc.} = 398.0$$

$$0.08 \text{ of same} = \underline{31.8}$$

$$P_{N_2} \text{ at 50 cc.} = 366.2$$

$$\text{Per cent CO}_2 = 100 \times \frac{463.4 - 463.0}{463.4} = 0.09$$

$$\text{Per cent O}_2 = 100 \times \frac{463.0 - 366.2}{463.4} = 20.89$$

$$\text{Per cent N}_2 = 100 \times \frac{366.2}{463.4} = 79.02$$

#### *Determining the c Correction*

After the  $\text{CO}_2$  has been absorbed the pressure exerted by the residual  $\text{O}_2 + \text{N}_2$  at 49 cc. volume would be inexactly calculated as  $p_2 - p_0$ . The manometer reading without any gas present, but with the 1 cc. of  $\text{NaOH}$  solution in the chamber, would be slightly *higher* than the  $p_0$  observed at the beginning of the analysis, with neither gas nor solution in the chamber. The weight of the short column of solution in the chamber presses on the mercury there, and in consequence raises the height of the mercury column in the manometer tube required to balance the meniscus in the chamber at the 50 cc. mark. The  $c$  correction required for this effect varies somewhat with the shape of the bottom of the chamber and the consequent height of the column of 1 cc. of solution. The value of  $c$  is, however, usually in the neighborhood of 1.5 mm.

The  $c$  correction is determined as follows: The  $p_0$  point is determined, with the chamber free of both gas and visible amounts of water, as described on p. 473. Then 1 cc. of 1 N sodium hydroxide is admitted, as described for absorption of  $\text{CO}_2$ , the chamber is evacuated till the mercury falls to the 50 cc. mark, and the air is extracted from the solution by shaking the latter 1 minute. The extracted small bubble of gas is ejected from the top of the chamber without loss of solution. The chamber is again

evacuated and the mercury meniscus is brought to the 50 cc. mark. The manometer is again read. This reading is  $p_0 + c$ .

The difference between it and the  $p_0$  reading is the  $c$  correction. To determine  $c$  within 0.1 mm. one makes several check readings of  $p_0$  with the chamber empty and also several of  $p_0 + c$ , with the 1 cc. of solution in the chamber. Once determined, the  $c$  correction serves for all analyses in which the same chamber is used.

### *Temperature Correction*

If, during the interval between the  $P_S$  and the  $P_{O_2 + N_2}$  or  $P_{N_2}$  measurement, changes exceeding  $0.1^\circ$  from the temperature of the  $P_S$  measurement are noted in the thermometer of the water jacket, the value of  $P_{O_2 + N_2}$  or  $P_{N_2}$  is calculated by Equation 10 or 11 instead of by Equation 4 or 6.

$$(11) \quad \text{Corrected } P_{O_2 + N_2} = 0.98 (p_2 - [p_0 + c] + 1.3 [t_0 - t_2]) \times \frac{T_0}{T_2}$$

$$(12) \quad \text{Corrected } P_{N_2} = 0.92 (p_3 - p_4) \times \frac{T_0}{T_4}$$

$T_0$ ,  $T_2$ , and  $T_4$  = values of absolute temperature at the readings  $p_0$ ,  $p_2$ , and  $p_4$ ;  $t_0$  and  $t_2$  = temperature in degrees centigrade at readings  $p_0$  and  $p_2$ . The values of the ratio,  $T_0:T_2$ , or  $T_0:T_4$ , for ordinary temperature changes are given below Equation 1 on p. 471. In Equation 11 the term,  $1.3 (t_0 - t_2)$ , serves to correct for the vapor pressure change, of about 1.3 mm. per  $1^\circ$ , caused by the temperature change,  $t_0 - t_2$ . Equation 11 covers the temperature correction in an exceptional case, where an intermediate operation (absorption of CO<sub>2</sub>) may allow time for a change of temperature between the two readings ( $p_0$  and  $p_2$ ) on which a single gas measurement ( $P_{O_2 + N_2}$ ) depends.

### *Necessity for Accuracy in Measurement of Volumes of Alkali and Hyposulfite Solutions Added*

The accuracy with which the volumes 49 and 46 cc. are defined, at which the pressures of residual gases are measured after absorption of CO<sub>2</sub> and O<sub>2</sub> respectively, is determined by the accuracy

with which the 1 and 3 cc. portions of alkali and hyposulfite solution, respectively, are measured into the chamber. An error of 0.01 cc. in the measurement of either solution would cause an error of 1 part in 5000 in the volume of gas space at which the pressure is measured. With calibrated stop-cock pipettes, however, it is simple to make the deliveries of solution into the chamber with errors less than 0.01 cc.

TABLE I

*Manometric Analysis of Gas Mixtures for Carbon Dioxide, Oxygen, and Nitrogen by Simple Absorption*

Pressure measurements at 50 cc. volume

Sample No.	Manometric analysis by Method A with approximately 35 cc. samples			Haldane analysis, with approximately 10 cc. samples		
	CO <sub>2</sub>	O <sub>2</sub>	N <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>	N <sub>2</sub>
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	5.59	19.68	74.73	5.55	19.72	74.73
	5.53	19.75	74.72	5.57	19.70	74.73
	5.49	19.70	74.81			
	5.59	19.66	74.75			
	5.61	19.74	74.65			
2	10.36			10.42		
	10.45			10.43		
3	6.66	19.46	73.88	6.70	19.46	73.84
	6.70	19.38	73.92	6.71	19.49	73.80
4	3.98	19.91	76.11	4.01	19.93	76.06
	3.88	19.96	76.16	4.00	19.98	76.02
	3.95	20.07	75.98			

Similar accuracy in the calibration of the exact total gas volume held above the 50 cc. mark is not necessary. Any error in this calibration will have so nearly a proportional effect on pressure measurements at 49 and 46 cc., that an error of 0.1 cc. in the 50 cc. calibration is required to affect oxygen results by 1 part in 5000.

Table I shows results obtained with the technique outlined above



compared with those yielded by the standard Haldane (1912) method for air analysis.

**METHOD B. NITROGEN IN AIR, OR IN MIXTURES OF CO<sub>2</sub>, O<sub>2</sub>, AND N<sub>2</sub> WITH OVER 10 PER CENT OF N<sub>2</sub>**

In some cases, as in the lung volume determinations by the nitrogen dilution method used by Lundsgaard and Van Slyke

TABLE II

*Determination of Nitrogen in Atmospheric Air and in Air Diluted with Oxygen. Simple Absorption of Carbon Dioxide and Oxygen by Method B*

Pressure measurements at 50 cc. volume

Sample No.	Observations						Calculations			
	$P_0$	$P_1$	Temper-	$P_2$	$P_3$	Temper-	$P_S = P_1 - P_0$	$P_{N_2}$		Per cent N <sub>2</sub> = 100 $\times \frac{P_{N_2}}{P_S}$
	Chamber empty	Sample in chamber	ature at $P_S$ measurement	N <sub>2</sub> + Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub> in chamber	Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub> only in chamber	ature at $P_{N_2}$ measurement		0.94 ( $p_2 - p_1$ ) uncorrected	Corrected to temperature of $P_S$	
	mm.	mm.	°C.	mm.	mm.	°C.	mm.	mm.	mm.	
1	89.5	609.7	26.5	526.0	89.0	26.7	520.2	410.7	410.5	78.91*
2	90.6	617.2	27.2	533.1	90.1	27.4	526.6	416.3	416.1	78.92*
3	88.6	617.2	26.4	532.6	88.2	26.6	528.4	417.8	417.6	79.03*
4	87.0	624.0	24.5	538.4	86.7	24.7	537.0	424.7	424.5	79.05*
5	85.7	617.0	25.0	531.6	84.9	25.2	531.3	419.8	419.6	79.01*
6	85.4	487.8	25.0	239.7	84.7	25.2	402.4	145.6	145.5	36.13
	85.4	488.4	25.2	240.0	84.6	25.4	403.0	146.0	145.9	36.21
7	84.4	497.8	24.0	205.2	83.9	24.2	413.4	114.0	113.9	27.53
	84.6	494.3	24.6	204.4	84.5	24.9	409.7	112.6	112.5	27.47
8	85.7	496.8	24.9	233.0	84.6	25.1	411.1	139.4	139.3	33.90
	85.2	487.5	25.0	229.8	84.7	25.2	402.3	136.4	136.3	33.90
9	84.3	498.8	24.1	201.4	83.5	24.3	414.5	110.8	110.7	26.72
	84.0	486.9	23.7	197.9	83.3	24.0	402.9	107.7	107.6	26.72

\* Atmospheric air. Nitrogen content = 79.04 per cent.

(1918), the nitrogen content of the air is the only figure desired. In this case the analysis is simplified by absorbing the CO<sub>2</sub> and O<sub>2</sub> together by the alkaline hyposulfite.

The sample is measured as in the preceding analysis.

$$P_S = p_1 - p_0$$

Then 3 cc. of the hyposulfite solution are introduced into the chamber, as above described, and the  $O_2$  and  $CO_2$  are absorbed together by 3 minutes shaking. Reading  $p_2$  is taken with the mercury meniscus at the 50 cc. mark. The gases are then ejected without loss of solution and reading  $p_3$  is taken.

All readings being taken with the *mercury* meniscus in the chamber at the 50 cc. mark, the calculations resemble those of the preceding analysis.

$$P_{N_2} = 0.94 (p_2 - p_3)$$

$$\text{Per cent } N_2 = 100 \times \frac{P_{N_2}}{P_S}$$

$$\text{Per cent } (CO_2 + O_2) = 100 - \text{per cent } N_2$$

Table II indicates the results for  $N_2$  obtained by the above method. Samples 1 to 5 were those of atmospheric air, which should contain 79.04 per cent of  $N_2$ . Samples 6 to 9 were mixtures of respired air and oxygen analyzed in the course of lung volume determinations.

#### METHOD C. SMALL AMOUNTS OF $N_2$ OR OTHER INERT GAS PRESENT AS IMPURITY IN $O_2$ OR $CO_2$

In case one wishes to determine traces of  $N_2$  as impurity in  $CO_2$  or  $O_2$ , the unabsorbed  $N_2$  is measured by its pressure at 2 or 0.5 cc. volume. *In such analyses it is necessary to use gas-free hyposulfite solution kept over mercury*, because when the oxygen has been absorbed the contents of the chamber are under a high vacuum, and dissolved  $N_2$  present in the hyposulfite solution would escape into the gas phase in sufficient amount to affect the results.  $P_S$  is measured as  $p_1 - p_0$ , in the manner described above. Then the  $O_2$  and  $CO_2$  are absorbed with air-free hyposulfite, and  $p_2$  is read with the unabsorbed gas (*solution* meniscus) at either 2 or 0.5 cc. volume. The gas is then ejected without loss of solution and  $p_3$  is read, with the chamber free of gas and the solution meniscus at the same mark as for the  $p_2$  reading.

$$P_{N_2} = p_2 - p_3$$

$$\text{Per cent N}_2 = 100 \times \frac{a}{A} \times \frac{P_{N_2}}{P_S}$$

$a$  is the small volume, 0.5 or 2 cc., at which  $P_{N_2}$  is measured, and  $A$  is the total chamber volume, 50 cc. in the usual chamber, at which  $P_S$  is measured. For chambers of the ordinary dimensions,

TABLE III  
*Determination of Nitrogen (or Hydrogen) as an Impurity in Oxygen by Method C.  $P_S$  at 50 Cc.*

Tank No.	Observations							Calculations				
	$P_0$ Chamber empty	$P_1$ Sample in chamber	Temperature at $P_S$ measurement	$P_2$ $N_2 + Na_2SO_4$ in chamber	$P_3$ $Na_2SO_4$ only in chamber	Temperature at $P_{N_2}$ measurement	Gas volume $a$ at $P_{N_2}$ measurement	$P_S = p_1 - p_0$	$P_{N_2}$		Per cent $N_2$	Formula for calculation of per cent $N_2$
	mm.	mm.	°C.	mm.	mm.	°C.	cc.	mm.	$= p_2 - p_1$ uncorrected	Corrected for temperature change from $P_S$		
1	75.6	479.8	21.8	305.3	149.1	22.4	0.5	404.2	156.2	155.9	0.386	$\frac{P_{N_2}}{P_S}$
	76.9	307.4	22.8	241.4	150.9	23.2	0.5	230.5	90.5	90.4	0.392	$\frac{P_{N_2}}{P_S}$
2	77.6	485.8	23.2	326.5	150.5	24.0	0.5	408.2	176.0	175.5	0.430	"
	78.4	482.8	24.0	325.3	150.3	24.5	0.5	404.4	175.0	174.7	0.432	"
3	89.1	487.0	24.0	229.6	172.8	24.4	2.0	397.9	56.8	56.7	0.570	$\frac{4 P_{N_2}}{P_S}$
	89.7	485.7	24.4	227.4	172.0	24.9	2.0	396.0	55.4	55.3	0.559	$\frac{4 P_{N_2}}{P_S}$

with  $A = 50$  cc. and  $a = 0.5$  or 2 cc., the calculations simplify to the following.

$$\text{Per cent N}_2 = \frac{P_{N_2}}{P_S} \text{ when } a = 0.5 \text{ cc. or}$$

$$\text{Per cent N}_2 = \frac{4 P_{N_2}}{P_S} \text{ when } a = 2 \text{ cc.}^1$$

<sup>1</sup> If, on checking the calibration of  $a$  with water,  $a'$  the actual volume is found to be different from the supposed gas volume (0.5 or 2 cc.), the observed pressure  $P_{N_2}$  must here be corrected by multiplication by  $\frac{a'}{a}$ .

The gas thus calculated as "N<sub>2</sub>" may be N<sub>2</sub>, H<sub>2</sub>, or any other gas which does not combine with alkali or hyposulfite.

Table III indicates the results obtained when the contents of three tanks of commercial oxygen gas were thus analyzed for inert impurities.

#### SUMMARY

Methods are described for the determination of carbon dioxide, oxygen, and nitrogen in gas mixtures by simple absorption in the manometric apparatus originally designed by Van Slyke and Neill for blood gases.

The gas sample and the nitrogen are measured by the pressures they exert at defined volumes. Carbon dioxide and oxygen are measured by successively absorbing them with alkali and hyposulfite solution, respectively, in the chamber of the manometric apparatus, and noting the resultant decreases in pressure.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

### II. CARBON DIOXIDE BY THE ISOLATION METHOD

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In this paper there is described a procedure\* whereby the CO<sub>2</sub> in a gas mixture is first isolated from other gases by absorption with alkali solution in the chamber of the manometric apparatus. The other gases are then ejected, the absorbed CO<sub>2</sub> is set free by acid, and is determined as in blood analyses. By this procedure the CO<sub>2</sub> in any desired volume of gas can be absorbed, and then set free and determined by the pressure it exerts at 2 or 0.5 cc. volume. The error with the usual manometric chamber, is, as in blood gas determination, less than 1 per cent of the amount of CO<sub>2</sub> determined, and could doubtless be further reduced by modifying the manometric chamber. Thus Van Slyke, Hastings, Heidelberger, and Neill (1922), using a chamber of 100 instead of 50 cc. total volume, with measurements of CO<sub>2</sub> pressures at 5 instead of 2 cc. volume, limited the error of blood CO<sub>2</sub> determinations to about 1 part in 500. The analyses published in this paper were obtained with the usual 50 cc. chamber, and therefore do not represent the maximum accuracy that could be obtained by modifying the chamber for that purpose.

A carbon dioxide method in which, as in this, the error in terms of per cent of the total gas mixture diminishes in proportion to the CO<sub>2</sub> content, is especially adapted to analysis of gas mixtures containing small amounts of carbon dioxide. With the isolation method, and the usual 50 cc. manometric chamber, one can readily determine the CO<sub>2</sub> content of atmospheric air to 0.0003 volume

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per cent, or 0.01 of the amount present. Such precision with apparatus modeled on the usual principles of gas analysis can be obtained only with most elaborate precautions, because it would involve measurement of total gas volumes with an accuracy of 1 part in 300,000.

The method serves well for determination of carbon dioxide in expired or alveolar air, where the CO<sub>2</sub> content runs from 6 to 2 per cent. The error, with the usual manometric chamber employed, is within 1 per cent of the amounts of CO<sub>2</sub> determined.

In analyses of gases with more than 10 or 15 per cent of CO<sub>2</sub>, the simple absorption method, described in the preceding paper (Van Slyke and Sendroy, 1932), is preferable, unless it is desirable to use minimal gas samples. In that case the micro form of the method here described can be used, with samples of only 1 to 1.5 cc.

### *Reagents*

*5 N Sodium Hydroxide, Approximate.*

*1 N Hydrochloric Acid, Approximate*—83 cc. of concentrated hydrochloric acid of 1.19 specific gravity diluted to a liter.

*0.1 N Sodium Hydroxide, Approximate, of Minimal CO<sub>2</sub> Content*—Sodium hydroxide is dissolved in an equal volume of water and the solution is let stand till the nearly insoluble Na<sub>2</sub>CO<sub>3</sub> has settled. Of the supernatant solution, 6 cc. are pipetted into 1 liter of water, which has been freed of CO<sub>2</sub> by adding a drop of concentrated hydrochloric acid and boiling. About 1 cc. of 1 per cent alizarin red solution is added. The 0.1 N alkali solution is immediately poured into 50 cc. flasks or bottles closed with paraffined corks or vaselined glass stoppers. After one of these flasks has been opened to use part of the solution, the residue is thrown away. As an alternative method of preservation larger amounts of the alkali may be kept in closed air-free containers over mercury, as shown in Fig. 6 of Van Slyke and Neill (1924).

### *Apparatus*

The only special apparatus besides the manometric is a 25 cc. burette for holding CO<sub>2</sub>-free NaOH solution. The tip of the burette is provided with a rubber ring and must be long enough to fit into the cup of the manometric chamber, as shown in Fig. 3 of Van Slyke (1927). The top of the burette is closed by a 1-hole

stopper into which is fitted a soda-lime tube to prevent entrance of atmospheric  $\text{CO}_2$ . When the burette is not in use the outlet is kept immersed in mercury to minimize absorption of atmospheric  $\text{CO}_2$  by the alkali at the tip.

METHOD A, FOR GAS SAMPLES NOT EXCEEDING THE VOLUME OF THE  
MANOMETRIC CHAMBER

In this method the sample is measured in one portion by the pressure it exerts at 50 or 2 cc. volume in the chamber. It is the method of choice when the gas analyzed contains over 1 (and less than 10) per cent of  $\text{CO}_2$ , or when the  $\text{CO}_2$  content is less than 1 per cent and maximal accuracy is not essential. When the  $\text{CO}_2$  content is less than 1 per cent, Method B, described later in this paper, is more exact, but it is somewhat less rapid.

It is desirable that the analyst familiarize himself with the introductory sections on general technique in Paper I of this series (Van Slyke and Sendroy, 1932).

*Introduction and Measurement of Sample*

*Macro Samples*—Before the gas sample is admitted to the chamber one estimates the approximate pressure in mm. which a sample of desirable size will exert at 50 cc. volume. A desirable sample will contain 0.5 to 1.0 cc. of  $\text{CO}_2$ , which will give a pressure of 200 to 400 mm. at 2 cc. volume. A simple rule to follow to calculate the pressure which a sample of desired size will exert at 50 cc. is to divide 1200 by the expected percentage of  $\text{CO}_2$  in the gas. *E.g.*, if alveolar air, with probably 6 per cent  $\text{CO}_2$ , is analyzed, a sample is taken which will give a  $P_s$  of  $\frac{1200}{6} = 200$  mm. pressure at 50 cc. The  $\text{CO}_2$  in this sample will then exert  $0.06 \times 200 = 12$  mm. pressure at 50 cc., and 25 times as much, or 300 mm. at 2 cc. when  $P_{\text{CO}_2}$  is determined in the final measurement. When expired air from a Tissot spirometer, with a  $\text{CO}_2$  content of usually about 4 per cent is analyzed, one takes sufficient sample to give a  $P_s$  of about 300 mm. If gas of less than 1.5 per cent  $\text{CO}_2$  content is analyzed, as large a sample as possible is taken; enough to give a  $P_s$  of 500 mm.

Before the sample is admitted the chamber is washed with acidulated water, which is ejected by slow admission of mercury.



The  $p_0$  reading is then taken, with the chamber empty of gas and the mercury at the 50 cc. mark. The sample of desired size is then admitted as described for "Admission of sample regulated by pressure" in the preceding paper (Van Slyke and Sendroy, 1932). The  $p_1$  reading is then taken with the mercury meniscus again at the 50 cc. mark.

$$P_S = p_1 - p_0$$

*Micro Samples*—A sample of about 1.5 cc. volume at atmospheric pressure is admitted as described by Van Slyke and Sendroy (1932) for "Admission of sample regulated by volume." The readings of  $p_0$  and  $p_1$  in this case are taken with the mercury in the chamber at the 2 instead of the 50 cc. mark.

#### *Absorption of CO<sub>2</sub> from Gas Sample*

After the gas sample has been measured, 3.0 cc. of the CO<sub>2</sub>-free 0.1 N sodium hydroxide solution, measured to 0.1 cc., are admitted into the chamber from the soda-lime guarded burette. The addition of alkali is made in the manner described for "Quantitative transfer of solution to the chamber without washing" in a previous paper (Van Slyke, 1927). Before inserting the burette tip into the cup of the chamber, 0.5 cc. of the solution is wasted, in order to remove from the tip the drop which may have absorbed CO<sub>2</sub> from the air.

After admission of the alkali, the mercury in the chamber is lowered until only the lower third of the chamber is filled with the metal. The chamber is then shaken rather slowly for 2 minutes. This causes complete absorption of the CO<sub>2</sub> by the alkali, which is thrown about on top of the mercury in such a way that it comes into thorough contact with the gas.

The residual gases are then ejected. It is not necessary that the last few c.mm. of gas be ejected, but it is essential that none of the alkali solution rise into the cup. The ejection of gas is therefore stopped when the alkali solution has entered the bore of the stop-cock.

After ejection of unabsorbed gases, 1 cc. of the 1 N hydrochloric acid is placed in the cup, and 0.5 cc. is run into the chamber. The CO<sub>2</sub> is now determined as described for blood analyses (Van Slyke and Neill, 1924). The stop-cock of the chamber is sealed with

mercury and the liberated  $\text{CO}_2$  is extracted by 2 minutes shaking at the 50 cc. mark. The reading  $p_2$  is then taken, with the precautions for this measurement given by Van Slyke and Neill (1924, p. 533).

The reading of the gas pressure  $p_3$  is taken with 2 cc. of gas volume, unless so little  $\text{CO}_2$  is present that the  $P_{\text{CO}_2}$  at 2 cc. volume is less than 100 mm. In this case the reading is taken with the gas at 0.5 cc. volume.

The stop-cock controlling the mercury in the chamber is opened, and the  $\text{CO}_2$  is absorbed with 0.3 cc. of 5 N sodium hydroxide solution, as described on p. 546, of Van Slyke and Neill. Then reading  $p_3$  is taken with the same gas volume as at the  $p_2$  reading. The alizarin indicator serves to show that the entire solution in the chamber turns alkaline.

A *blank analysis* is performed, in which no gas is admitted to the chamber. The pressure fall observed when the 5 N sodium hydroxide is added is the  $c$  correction. It should not exceed 4 to 6 mm. with the gas at 2 cc. volume if the 0.1 N alkali solution has been prepared and handled with the above outlined precautions to minimize its  $\text{CO}_2$  content.

$$P_{\text{CO}_2} = p_2 - p_3 - c$$

### Calculation

The  $\text{CO}_2$  content of the gas is calculated as:

$$\text{Volumes per cent } \text{CO}_2 = \frac{P_{\text{CO}_2}}{P_S} \times \text{factor}$$

The values of the factor are given in Table I.

The factors of Table I are calculated as follows: The volume,  $V_{\text{CO}_2}$ , of  $\text{CO}_2$ , in cc., reduced to  $0^\circ$ , 760 mm., present in the sample, is calculated by multiplying  $P_{\text{CO}_2}$  by the factor,  $f_1$ , which is derived from Equation 4 of Van Slyke and Neill (1924).

$$f_1 = \frac{i a}{760 (1 + 0.00384i)} \left( 1 + \frac{S \alpha'}{A - S} \right)$$

$A$  = total volume of chamber at lower mark = 50 cc. in present chamber;  $S$  = volume of solution from which the  $\text{CO}_2$  is extracted = 3.5 cc. in this analysis;  $a$  = volume at which the pressure of

the extracted CO<sub>2</sub> gas is measured = 2 or 0.5 cc.;  $t$  = temperature centigrade;  $\alpha'$  is the distribution coefficient of CO<sub>2</sub> between

TABLE I  
*Factors by Which Ratio,  $PCO_2:P_S$ , Is Multiplied in Order to Calculate Volume Per Cent of CO<sub>2</sub>*

Temperature °C.	Factors when sample pressure is taken at 50 cc. volume		Factors when sample pressure is taken at 2 cc. volume	
	$PCO_2$ measured with gas at 2 cc. volume	$PCO_2$ measured with gas at 0.5 cc. volume	$PCO_2$ measured with gas at 2 cc. volume	$PCO_2$ measured with gas at 0.5 cc. volume
10	4.444	1.132	111.1	28.29
11	32	29	110.8	22
12	21	26	0.5	15
13	11	24	0.3	09
14	02	22	0.0	04
15	4.393	20	109.8	27.98
16	83	17	9.6	92
17	73	15	9.3	87
18	65	13	9.1	82
19	58	12	9.0	77
20	51	10	8.8	72
21	44	08	8.6	68
22	37	06	8.4	63
23	30	04	8.3	59
24	24	02	8.1	54
25	18	00	7.9	50
26	12	1.099	7.8	47
27	06	97	7.6	44
28	00	96	7.5	41
29	4.295	95	7.4	38
30	91	94	7.3	34
31	86	93	7.2	31
32	82	92	7.0	27
33	77	91	6.9	24
34	73	90	6.8	20

the gaseous and aqueous phases;  $i$  = factor correcting for re-absorption of CO<sub>2</sub> while the volume of the extracted gas is being

diminished from  $A - S$  to  $a$  cc. The values of  $i$  used are those found by Van Slyke and Sendroy (1927); *viz.*, 1.017 when  $P_{\text{CO}_2}$  is measured at 2 cc. volume, and 1.037 when  $P_{\text{CO}_2}$  is measured at 0.5 cc. volume. (Values of  $100 f_1$  are given in Table IV.)

The volume,  $V_s$ , of the sample, in cc. reduced to  $0^\circ$ , 760 mm., is calculated by multiplying  $P_s$  by  $f_2$ .

$$f_2 = \frac{a}{760 (1 + 0.00384t)}$$

The symbols,  $a$  and  $t$ , have the same significance as above. The coefficient 0.00384 instead of the usual 0.00367 is used in the denominator in order to correct for expansion of the mercury in the manometer with temperature, as discussed by Van Slyke and Neill (1924) on p. 540 of their paper. The calculation of  $V_s$  as  $V_s = f_2 P_s$  is an application of the general formula used for reductions of gases to standard conditions; *viz.*,

$$\text{Volume of gas at } 0^\circ, 760 \text{ mm.} = (\text{volume at } t^\circ, P \text{ mm.}) \times \frac{P}{760 (1 + 0.00384t)}$$

In the present case, the volume at  $t^\circ$ ,  $P$  mm., is  $a$ , the volume of the gas in the chamber when the manometer is read.

The volume per cent of  $\text{CO}_2$  in the sample is calculated as:

$$\text{Volume per cent } \text{CO}_2 = \frac{100 \times V_{\text{CO}_2}}{V_s} = \frac{100 f_1 P_{\text{CO}_2}}{f_2 P_s} = \frac{P_{\text{CO}_2}}{P_s} \times \text{factor}$$

The factor is  $\frac{100 f_1}{f_2}$ , values of which are given in Table I.

Table II indicates the results obtained by the macro analysis of gas mixtures of varying  $\text{CO}_2$  content (from 0.03 to 10 per cent), by the method of isolation described in this section. The results are compared with those obtained by the usual method of Haldane analysis. In the case of Samples 2 and 3 in Table I, these mixtures, because of their small  $\text{CO}_2$  content could not be accurately analyzed in the Haldane apparatus. The values given are those calculated by the addition of  $\text{CO}_2$ -free air to known, measured volumes of pure  $\text{CO}_2$ .

TABLE II

*Determinations of CO<sub>2</sub> in Air by Method A, in Which Both Sample and Isolated CO<sub>2</sub> Are Measured by Pressure*

Macro samples measured by pressure at 50 cc. volume

	Sample No.	Manometric analysis		Haldane analysis for CO <sub>2</sub> , 9 to 10 cc. samples
		Approximate size of sample in terms of $P_S$ at 50 cc.	CO <sub>2</sub> content found	
Air of 0.03 to 1 per cent CO <sub>2</sub> content. $P_S$ at 50 cc., $P_{CO_2}$ at 0.5 cc.	1	150	per cent 0.90	per cent 0.88
	2	520	0.90 0.031 0.031 0.031	0.90 0.033*
	3	500	0.031 0.079 0.077 0.078 0.080	0.076*
Air of 3 to 10 per cent CO <sub>2</sub> content. $P_S$ at 50 cc., $P_{CO_2}$ at 2 cc.	4	160	3.25	3.32
	5	170	3.30 5.31	3.32 5.21
	6	170	5.25 10.75	5.25 10.70
	7	150	10.61 3.89 3.92	10.71 3.87 3.86
	8	140	3.89 6.72 6.71	3.87 6.78 6.78
	9	170	6.78 9.94 10.01 9.90 9.93	6.78 10.00 9.99
	10	150	3.12	3.13 3.13
	11	150	3.05 3.05	3.00 3.03
	12	150	10.34 10.34 10.36	10.31 10.32
	13	150	6.98 7.00	6.98 6.97
	14	160	4.91 4.87	4.86 4.88

\* Mixture of known CO<sub>2</sub> content.

Table III covers the results of micro analyses with samples of 0.5 to 1.5 cc.

TABLE III

*Determinations of CO<sub>2</sub> in Air by Method A, in Which Both Sample and Isolated CO<sub>2</sub> Are Measured by Pressure*

Micro samples measured by pressure at 2 cc. volume

	Sample No.	Manometric analysis		Haldane analysis for CO <sub>2</sub> , approximately 10 cc. samples
		Approximate size of sample in terms of $P_S$ at 2 cc.	CO <sub>2</sub> content found	
Air of 17 to 25 per cent CO <sub>2</sub> content. $P_S$ at 2 cc., $P_{CO_2}$ at 0.5 cc.	15	450	<i>per cent</i> 25.70	<i>per cent</i> 25.58
			25.65	25.64
	16	400	17.62	25.69
			17.65	17.56
				17.63
				17.64
Air of 3 to 10 per cent CO <sub>2</sub> content. $P_S$ at 2 cc., $P_{CO_2}$ at 0.5 cc.	17	360-510	3.27	3.31
			3.28	3.35
			3.34	3.34
			3.34	
	18	370-390	6.63	6.56
			6.54	6.55
			6.60	6.57
			6.67	
			6.69	
			6.52	
	19	360-380	9.43	9.46
			9.38	9.46
			9.36	9.48
			9.41	
	20	370-390	6.44	6.53
			6.46	6.53
			6.45	
	21	370-380	5.89	5.88
			5.92	5.83
			5.91	5.87
	22	430	6.50	6.56
				6.58
	23	500	5.86	5.95
				5.97

*Example of Calculation*—Analysis of CO<sub>2</sub> in air mixture (Sample 14, Table II) gave the following.

Measured at 23.0° at the 50 cc.	Measured at 23.1° at the 2 cc.
mark	mark
mm.	mm.
$p_1 = 242.2$	$p_1 = 335.7$
$p_0 = 86.0$	$p_1 = 155.0$
$P_S = 156.2$	$p_1 - p_1 = 180.7$
	$c = 5.2$
	$P_{CO_2} = 175.5$

Multiplying  $\frac{P_{CO_2}}{P_S}$  by the appropriate factor, 4.330, from Table I, we obtain

$$\text{Volume per cent CO}_2 = \frac{175.5}{156.2} \times 4.330 = 4.87$$

*Corrections for Calibration Errors of Chamber and for Effect of Measuring  $P_S$  Over a Mercury Meniscus*—If  $a$ , the volume at which  $P_S$  is measured, is other than the 50 or 2 cc. assumed in calculating the factors of Table I, the observed  $P_S$  will have to be multiplied by the correction factor  $\frac{a}{2}$  or  $\frac{a}{50}$  to obtain the exact  $P_S$  for use with the factors of Table I. Similarly, if the volume at which  $P_{CO_2}$  is measured is other than the assumed 0.5 or 2.0 cc. the observed  $P_{CO_2}$  will require multiplication by  $\frac{a}{0.5}$  or  $\frac{a}{2}$  in order to obtain the exact  $P_{CO_2}$  for use with the factors of Table I.

In a well calibrated chamber the deviations from the assumed volumes will be negligible, except for  $P_S$  values, measured in the micro determinations, over a mercury meniscus at the 2 cc. mark. The chambers are calibrated for gas measurements over water menisci, and the gas space over a mercury meniscus at a given mark is greater than over a water meniscus at the same mark. If the bore of the chamber at the 2 cc. mark is 4 mm., as is generally the case, we have found that the gas space over a mercury meniscus at that mark is 0.012 cc. greater than over a water meniscus. The value of  $P_S$  measured at a 2 cc. mark exact for a water meniscus will then require multiplication by the correction factor  $\frac{2.012}{2.000} = 1.006$ , when the measurement is made over a mercury meniscus.

In general, if  $a$  represents the supposed gas volume (0.5, 2.0, or 50 cc.) held by the chamber over a water meniscus at a given mark,  $a'$  the actual volume above a water meniscus found in checking the calibration, and  $c_a$  the increase in gas volume measurement that results from changing from a water meniscus to a mercury meniscus, then the observed pressure,  $P_s$ , or  $P_{CO_2}$ , must be corrected by multiplication by  $\frac{a'}{a}$  if the pressure is observed with the gas over an aqueous meniscus, and by  $\frac{a' + c_a}{a}$  when the pressure is observed with the gas over a mercury meniscus. In the  $CO_2$  determinations in gas mixtures above described,  $\frac{a' + c_a}{a}$  is significant only in the measurement of  $P_s$  values at the 2 cc. mark for micro samples. For  $P_s$  measured with the gas at 50 cc. volume, as is the case except when larger samples are taken, the difference between mercury and water menisci is only enough to affect  $P_s$  by about 1 part per 1000, and may ordinarily be neglected.  $P_{CO_2}$  values in this analysis are all measured over water menisci, so that the  $c_a$  correction does not apply to  $P_{CO_2}$ .

#### METHOD B, FOR PRECISE DETERMINATION OF SMALL PROPORTIONS OF $CO_2$ IN AIR

The procedure is the same as the above, except that here a larger sample, 200 or 300 cc. of air, is shaken in successive portions with alkali in the manometric chamber, the absorbed  $CO_2$  being then extracted from solution and measured as above. The 0.03 per cent of  $CO_2$  in ordinary outdoor air can thus be measured with an accuracy of 1 part per 100.

The sample is measured by volume in a container of the type shown on the left in Fig. 1. The container, of, for example, 250 cc. volume, is calibrated by weighing it first empty, except for a film of water on the inner wall, and then with the bulb between the two cocks filled with water. The container, of which the inner walls should be moist, is connected with a mercury leveling bulb, and is completely filled with a sample of the air, the mercury being withdrawn as far as the lower cock. The container is then immersed in a water bath at a temperature about  $1^\circ$  higher than



that of the room, and after thermal equilibrium has been reached, the top stop-cock is exposed to the air and opened for a moment to release the internal pressure. The barometric pressure and the temperature of the water bath are recorded.

The container is then connected to the manometric chamber as shown in Fig. 1 by a flexible rubber tube of about 2 mm. bore and just sufficient length to permit the chamber to be shaken without

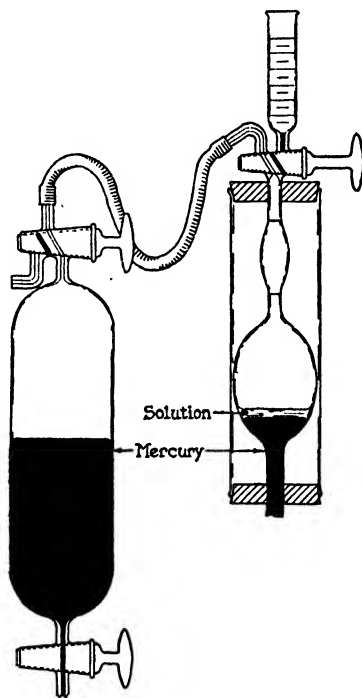


FIG. 1. Method of sampling and analysis of large volumes of gas with the manometric chamber.

disturbing the container. The connecting tube is then filled with mercury from the chamber, and about 35 cc. of the sample are run into the chamber. 3 cc. of the approximately CO<sub>2</sub>-free 0.1 N sodium hydroxide are then admitted to the chamber as above described, and the CO<sub>2</sub> in the air is absorbed by shaking slowly for 4 minutes, with 10 or 15 cc. of mercury in the chamber. The unabsorbed gas, except for a small bubble, is then ejected. Then

another portion of the sample is admitted and its  $\text{CO}_2$  is absorbed in the same manner. This procedure is repeated until all the gas from the calibrated container has passed through the chamber and has been shaken with the alkali. When the last portion of gas is run into the chamber a little mercury from the container is permitted to follow and fill the left-hand bore of the cock of the chamber.

After the  $\text{CO}_2$  from the last portion of air has been absorbed, the unabsorbed air is ejected without loss of solution, and 0.5 cc. of 1 *N* hydrochloric acid is admitted to the chamber. The  $\text{CO}_2$  is extracted from the solution, and  $P_{\text{CO}_2}$  is measured as directed for Method A. Manometer reading  $p_1$  is taken with the  $\text{CO}_2$  gas at 0.5 or 2.0 cc. volume, according to the amount present, and  $p_2$  with the residual gas at the same volume after the  $\text{CO}_2$  has been absorbed with 5 *N* alkali. The value of the  $c$  correction is determined in a blank analysis, in which no air is run through the chamber.

### Calculation

$$P_{\text{CO}_2} = p_1 - p_2 - c$$

$$\text{Volume per cent CO}_2 \text{ in air} = \frac{P_{\text{CO}_2} \times 100 f_1}{C \times f_2}$$

$C$  is the capacity in cc. of the container in which the gas sample was measured by volume.

$100 f_1$  is the factor from Table IV, by which  $P_{\text{CO}_2}$  is multiplied in order to obtain 100 times the cc. of  $\text{CO}_2$  present, reduced to  $0^\circ$ , at 760 mm.

$f_2$  is the usual factor,  $\frac{B - W}{760 (1 + 0.00367t)}$ , by which the observed volume of a moist gas is multiplied to obtain the volume reduced to  $0^\circ$ , 760 mm.  $B$  = barometric pressure, corrected for temperature,  $W$  = vapor tension of water at the temperature,  $t^\circ$  centigrade, at which the gas volume is observed. The values of  $f_2$  are not given here, as they are found in any text-book of gas analysis or of physicochemical tables. In place of values of  $\frac{1}{1 + 0.00367t}$  and values of  $B$  corrected for temperature, one may use uncorrected

values of  $B$  with the factor  $\frac{1}{1 + 0.00384t}$ . The use of the coefficient 0.00384 instead of 0.00367 makes a sufficiently exact correction.

TABLE IV

*Values of Factor, 100  $f$ , by Which  $P_{CO_2}$  Is Multiplied to Obtain 100  $V_{CO_2}$ ; for Use in Calculating Results from Method B*

Temperature	Factor when $P_{CO_2}$ is measured with gas at 2 cc. volume	Factor when $P_{CO_2}$ is measured with gas at 0.5 cc. volume
°C.		
10	0.2818	0.0718
11	00	14
12	0.2783	09
13	67	05
14	50	01
15	35	0.0697
16	19	93
17	04	89
18	0.2690	86
19	75	82
20	62	78
21	48	75
22	34	71
23	20	68
24	07	65
25	0.2594	61
26	81	58
27	69	55
28	57	52
29	45	49
30	33	46
31	22	43
32	11	40
33	00	37
34	0.2489	34

tion for the expansion of mercury in the barometer, whether the scale is glass or brass (see Van Slyke and Neill, 1924, p. 540).

*Examples of Calculation*—The nature of the results obtained in

analysis of atmospheric air, and of the calculations involved, are indicated by the data in Table V.

TABLE V

*Calculation of CO<sub>2</sub> Determined in Atmospheric Air by Method B, in Which Sample Is Measured by Volume from Separate Container*

Readings of  $p_1$  and  $p_2$  were made with gas at 0.5 cc. volume

	Analysis I	Analysis II
Measurement of samples		
$C$ , cc.....	291.9	268.6
$t$ , °C.....	24.3	24.2
$B$ , mm.....	765.2	765.2
$W$ , ".....	22.8	22.7
$f_1$ .....	0.897	0.897
Measurement of CO <sub>2</sub> pressures		
Temperature, °C...	23.3	21.8
$p_1$ , mm.....	256.0	270.0
$p_2$ , ".....	107.3	133.9
$p_1 - p_2$ , mm.....	148.7	136.1
$c$ , mm.....	13.6	13.6
$PCO_2$ , mm.....	135.1	122.5
100 $f_1$ .....	0.0667	0.0672
Calculations		
Volume per cent CO <sub>2</sub> .....	$\frac{135.1 \times 0.0667}{291.9 \times 0.897} = 0.0344$	$\frac{122.5 \times 0.0672}{268.6 \times 0.897} = 0.0342$

## SUMMARY

Carbon dioxide is isolated from other gases by shaking the gas mixtures in any desired volume with alkali solution in the chamber of the Van Slyke-Neill manometric apparatus. The other gases are ejected, and the CO<sub>2</sub> absorbed is set free with acid and determined, as in estimations of plasma CO<sub>2</sub> content.

The method is especially adapted to accurate determination of CO<sub>2</sub> when the latter is present in minimal proportions, as in atmospheric air, in which the CO<sub>2</sub> content can be estimated easily within 0.0003 volume per cent. In respired air the method gives results exact to within  $\pm 0.05$  volume per cent.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

### III. MANOMETRIC DETERMINATION OF CARBON DIOXIDE TENSION AND pH<sub>a</sub> OF BLOOD

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Hasselbalch, in 1916, showed that the pH of blood could be calculated by Henderson's mass law equation from the observed CO<sub>2</sub> tension and the CO<sub>2</sub> content. Difficulties attending determination of the CO<sub>2</sub> tension of blood as drawn, however, prevented the immediate application to blood analysis of this simple principle, which promised to obviate the technical difficulties of electrometric methods and the errors of colorimetric ones.

With modern refinements of the technique for CO<sub>2</sub> determinations in plasma, Eisenman (1926-27) solved the problem by determining the CO<sub>2</sub> absorption curve of the separated serum (curve with CO<sub>2</sub> tensions as abscissæ, CO<sub>2</sub> contents as ordinates), and interpolating on the curve the CO<sub>2</sub> content of the serum of the shed blood. The abscissa of the curve at the interpolated point indicates the CO<sub>2</sub> tension.

The present paper offers another procedure, which avoids the necessity of plotting a CO<sub>2</sub> absorption curve, and of depending upon interpolation. The CO<sub>2</sub> tension of blood is determined by equilibrating the blood at body temperature with a relatively small bubble of gas, a principle introduced by Pflüger (1872) and applied by Krogh (1908). Under the conditions used, the gas assumes the CO<sub>2</sub> tension of the blood, which is ascertained by determining the CO<sub>2</sub> content of the equilibrated bubble. In the present method the bubble is analyzed by the micro gas analysis

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described in the preceding paper (Van Slyke, Sendroy, and Liu, 1932).

A portion of the blood is then centrifuged, and the CO<sub>2</sub> content of the plasma is determined by the Van Slyke-Neill (1924) method. Since conditions of equilibration are such that the CO<sub>2</sub> content of the blood is not significantly changed, the blood used for equilibration can afterwards be used to supply the plasma for analysis. Because of the complications which the cells and the degree of oxygenation introduce by varying the value of  $pK'$  in the Henderson-Hasselbalch equation (Warburg, 1922; Van Slyke, Wu, and McLean, 1923), the pH calculation is greatly simplified by basing it on the CO<sub>2</sub> content of plasma or serum rather than on that of whole blood.

From the CO<sub>2</sub> tension and the plasma CO<sub>2</sub> content obtained, the plasma pH is calculated by the Henderson-Hasselbalch equation, with values for  $pK'$  and the solubility coefficient of CO<sub>2</sub> determined in previous papers from this laboratory (Hastings, Sendroy, and Van Slyke, 1928; Van Slyke, Sendroy, Hastings, and Neill, 1928). The deviation of the plasma pH values thus gasometrically determined, from values determined with the standard hydrogen electrode, has in none of our analyses exceeded 0.04 pH, and in the majority of analyses has not exceeded 0.02.

The results obtained by the procedure outlined give the acid-base balance of the blood plasma in terms of CO<sub>2</sub> tension, plasma pH, and CO<sub>2</sub> and bicarbonate contents. Sufficient blood remains for oxygen analyses, so that the factors most frequently sought in studies of the acid-base balance are obtained with the one blood portion of 9 cc.

In case conditions render impracticable the anaerobic centrifugation to obtain plasma for CO<sub>2</sub> determination, the CO<sub>2</sub> content and oxygenation may be determined in the whole blood, and from these values and the CO<sub>2</sub> tension the plasma CO<sub>2</sub> may be estimated, with an error not exceeding 2 volumes per cent, by means of the nomogram given in Fig. 2.

One factor of perhaps definite importance is neglected in this, as in all previous methods for routine determination of blood pH. Havard and Kerridge (1929) have reported that shed blood kept at 38° suffers, in a few minutes after drawing, a fall of 0.02 to 0.05 in pH. After this drop they found the pH to remain constant,

except for the slow fall that later sets in as the result of lactic acid formation from glucose. If the blood was cooled to  $18^{\circ}$  as soon as drawn, the initial pH fall required an hour and a half. These observations, made with glass electrodes, have been confirmed with hydrogen and quinhydrone electrodes by Laug (1930), who found a fall of 0.02 to 0.04 in the pH of plasma when the blood was kept at  $36^{\circ}$  for 13 minutes after being drawn. There is yet no explanation of the chemical cause of the slight but definite acidification. However, it appears that pH values found in plasma and serum by the techniques commonly applied, and by the one presented in this paper, are about 0.03 lower than the pH in the circulating blood. Such a limited error does not seriously impair the utility of the pH values in acid-base studies.\*

#### *Error Involved in Equilibration*

If oxygenated blood is equilibrated with air +  $\text{CO}_2$ , the  $\text{CO}_2$  tension in the gas will approach within 0.3 mm. the original  $\text{CO}_2$  tension of the blood, even if the original difference between blood and gas is as much as 10 mm. of  $\text{CO}_2$  tension. The degree of approximation is indicated by the following calculation.

If we assume that 1 cc. of gas is equilibrated with 9 cc. of normal blood, and that the initial  $\text{CO}_2$  tension of the gas phase is 40 mm., while that of the blood is 50 mm., the gas bubble, in order to raise its  $\text{CO}_2$  tension to 50 mm., will take from the blood 0.0126 cc. of  $\text{CO}_2$  (calculated at  $0^{\circ}$ , 760 mm.) and thereby reduce the blood  $\text{CO}_2$  content by 0.14 volume per cent. The fall in  $\text{CO}_2$  tension caused by removing the 0.0126 cc. of  $\text{CO}_2$  from the blood is about 0.3 mm. (see Fig. 91, p. 897, of Peters and Van Slyke, 1931).

The equilibration must be accomplished also without markedly changing the oxygenation of the hemoglobin in the blood, or the  $\text{CO}_2$  tension will be altered. Oxygenated hemoglobin acts as a stronger acid than reduced hemoglobin, so that increase in the  $\text{HbO}_2$  decomposes  $\text{BHCO}_3$  into  $\text{H}_2\text{CO}_3$ , with corresponding rise in the  $\text{CO}_2$  tension, and *vice versa* (for discussion of this phenomenon, see Peters and Van Slyke, p. 900 *et seq.*, 1931). However, when the gas bubble equilibrated with the blood is only  $\frac{1}{3}$  the volume of the latter, and the initial  $p_{\text{O}_2}$  of the blood is set at 80 mm. for arterial, and 25 mm. for venous blood, the changes in oxygenation of the



hemoglobin are too slight to affect seriously the CO<sub>2</sub> tension. This conclusion may be derived from the following calculation.

9 cc. of normal blood combine with about 1.80 cc. of O<sub>2</sub> to completely oxygenate the hemoglobin.<sup>1</sup> To change the  $p_{O_2}$  of the 1 cc. gas bubble by 10 mm., 0.0126 cc. of O<sub>2</sub> are required, which is enough to saturate 0.007 of the hemoglobin in the 9 cc. of blood. From Fig. 91, p. 897, of Peters and Van Slyke (1931), one can estimate that, with blood CO<sub>2</sub> content constant at 50 volumes per cent, change from complete oxygenation to complete reduction causes a decrease of 13 mm. in the CO<sub>2</sub> tension of blood. Hence, if the HbO<sub>2</sub> of the blood gives to the gas bubble, or the Hb takes from it, enough O<sub>2</sub> to change the  $p_{O_2}$  of the bubble 10 mm., the resultant change in blood CO<sub>2</sub> tension will approximate  $0.007 \times 13$  mm., or about 0.1 mm.

When the initial O<sub>2</sub> tension of the bubble is set at 80 mm. for arterial blood and 25 mm. for venous, it appears that oxygenation changes will rarely affect the CO<sub>2</sub> tension by more than 0.2 mm. (see Fig. 107, p. 987, of Peters and Van Slyke, 1931). In fact, the effect on  $p_{CO_2}$  of measured differences, up to 30 mm., between the initial oxygen tensions of the bubble and the blood has not been experimentally detectable (see Table III).

It appears that equilibration under the conditions used may be depended on to bring the CO<sub>2</sub> tension of the gas bubble within 0.5 mm. of the original CO<sub>2</sub> tension of the blood, and that when greater errors occur in blood  $p_{CO_2}$ , determined by the present method they are probably attributable to the micro analysis of the equilibrated gas bubble. This analysis is subject to a maximum error of about 0.2 volume per cent of CO<sub>2</sub>, equivalent to 1.5 mm. of CO<sub>2</sub> tension. The maximum error to be expected in the  $p_{CO_2}$  determination is therefore about  $0.5 + 1.5 = 2$  mm. This is in fact nearly the limit of error we have encountered, as seen in Tables I and II.

#### *Calculation of Plasma pH by Henderson-Hasselbalch Equation, and Sources of Error Involved*

This equation, its derivation from the law of mass action, and its various forms, have been discussed by Austin *et al.* (1922) and

<sup>1</sup> For brevity we shall use the symbols  $p_{CO_2}$  and  $p_{O_2}$  to indicate carbon dioxide tension and oxygen tension, respectively.

on p. 874 *et seq.* of Peters and Van Slyke (1931). The form in which it serves to calculate plasma or serum pH from the  $\text{CO}_2$  tension and total  $\text{CO}_2$  content of the fluid is expressed in Equations 1 and 2.

$$(1) \quad \text{pH}_s = 6.10 + \log \frac{[\text{CO}_2]_s - 0.067p_{\text{CO}_2}}{0.067p_{\text{CO}_2}}$$

when  $[\text{CO}_2]_s$  is expressed in volumes per cent of  $\text{CO}_2$  in the plasma or serum.

$$(2) \quad \text{pH}_s = 6.10 + \log \frac{[\text{CO}_2]_s - 0.0301p_{\text{CO}_2}}{0.0301p_{\text{CO}_2}}$$

when  $[\text{CO}_2]_s$  is expressed in millimols of  $\text{CO}_2$  per liter of plasma or serum.

$[\text{CO}_2]_s$  indicates the total  $\text{CO}_2$  content of the serum or plasma,  $p_{\text{CO}_2}$  the  $\text{CO}_2$  tension in mm. of mercury,  $\text{pH}_s$  the pH of serum or plasma.

These are special forms of the general Henderson-Hasselbalch equation

$$(3) \quad \text{pH} = \text{pK}' + \log \frac{[\text{HCO}_3]}{[\text{H}_2\text{CO}_3]}$$

In Equations 1 and 2  $\text{pK}'$  is represented by the constant, 6.10,  $\text{HCO}_3$  is calculated as  $[\text{total CO}_2] - [\text{H}_2\text{CO}_3]$ , and  $[\text{H}_2\text{CO}_3]$  is calculated as

$$(4) \quad \begin{aligned} \text{Volume per cent CO}_2 \text{ as H}_2\text{CO}_3 &= 100 \alpha \times \frac{p_{\text{CO}_2}}{760} \\ &= 0.067p, \text{ when } \alpha = 0.51 \end{aligned}$$

To calculate  $[\text{H}_2\text{CO}_3]$  in millimols per liter, the volume per cent factor is multiplied by 10, and divided by 22.26, the volume of 1 mol of  $\text{CO}_2$  at  $0^\circ$ , 760 mm. The factor 0.0301 is thus obtained.

It is evident that the precision of the gasometric  $\text{pH}_s$  depends upon the accuracy of four values; the  $[\text{CO}_2]_s$  and  $p_{\text{CO}_2}$  determined in the analysis, and the constants,  $\text{pK}'$  and  $\alpha$ . Each of these four values offers a possible source of error to the calculated  $\text{pH}_s$ . It appears that we may estimate these sources of error as follows:

*Solubility Coefficient of CO<sub>2</sub>*—The value of  $\alpha$  in normal human serum was found by Van Slyke, Sendroy, Hastings, and Neill (1928) to be 0.510, with variations only in the third decimal place. The presence of much lipoid in pathological serum, such as may occur in nephrosis, was found to increase the solubility, sometimes to as high as 0.54, because of the high solubility of CO<sub>2</sub> in fats and oils. However, even in such serum, it is probable that the value 0.510 represents approximately the solubility of CO<sub>2</sub> in the *water phase*, and that the use of the value,  $\alpha = 0.51$ , in calculating [H<sub>2</sub>CO<sub>3</sub>], seldom involves an error of over 1 per cent. An error of 1 part per 100 in [H<sub>2</sub>CO<sub>3</sub>] would alter the calculated pH<sub>s</sub> by 0.004.

*Total CO<sub>2</sub> Content of Plasma*—In the ordinary 50 cc. form of the Van Slyke-Neill apparatus, plasma CO<sub>2</sub> can be determined with an error not exceeding 1 part in 200. Such an error would affect calculated values of pH<sub>s</sub> by 0.002. If the plasma CO<sub>2</sub> content is not determined directly, but estimated from blood CO<sub>2</sub> by Fig. 2, the error in [CO<sub>2</sub>]<sub>s</sub> is increased to a possible (though unusual) maximum of 1 part in 20, and the resultant error in calculated pH<sub>s</sub> is raised to 0.02.

*CO<sub>2</sub> Tension*—When the blood CO<sub>2</sub> tension is determined by the method described in this paper, the maximum error, indicated by Tables I to III, is about 1 part in 20. Such an error in  $p_{\text{CO}_2}$  would cause an error of 0.02 in the calculated pH<sub>s</sub>.

*The Constant, pK'*— $pK'$  is not an absolute constant, but diminishes as the total electrolyte content of a solution, expressed in terms of ionic strength, increases. The relationship is indicated by the formula  $pK' = 6.33 - 0.5\sqrt{\mu}$  where  $\mu$  represents ionic strength (Hastings and Sendroy, 1925). The increase in electrolyte content caused by adding oxalate to blood should accordingly depress somewhat the  $pK'$  value of plasma below that of serum separated without addition of any electrolyte. The data of Cullen (1922), however, indicate a  $pK'$  for normal horse oxalated plasma only 0.003 below that for the serum of the same blood. An increase in the value of  $pK'$  may be expected in some pathological conditions (especially in severe nephritis) where the total electrolyte content of the plasma is subnormal, so that the use of a normal average  $pK'$  for such sera should give pH<sub>s</sub> results slightly too low. The data of Hastings, Sendroy, and Van Slyke (1928) show, in fact,  $pK'$  values in two nephritics higher by 0.02 units than the

average normal value. It is probable that, for serum and plasmas of abnormal electrolyte content, a correction for  $pK'$ , estimated from the deviation of total base from its normal value, could be applied. The magnitude of such correction could be estimated from the formula of Hastings and Sendroy quoted above. Its application to serum and plasma would need to be tested, however, before routine application.

Hastings, Sendroy, and Van Slyke (1928) found in sixteen determinations with human sera, of which six were nephritic, a total range of  $pK'$  values between 6.097 and 6.122 in normal sera, and between 6.108 and 6.134 in nephritic sera. The average of all the values was 6.105, and this agreed with the mean calculated from data of other authors reported since 1922. Accordingly 6.10 was taken as the value of serum  $pK'$ . This value for  $pK'$  has since been generally used, and is employed in calculation of gasometric pH values by the present method. From our data in Table IV, *B*, it can be seen that a  $pK'$  value of 6.11 would give gasometric pH values in slightly closer average agreement with the electrometric values, but the difference is not decisive enough to warrant the slight change from the  $pK'$  value established by the data quoted above.

According to the above considerations a gasometric pH<sub>s</sub> determination by the present method is subject to the following maximum errors from the four values on which the pH<sub>s</sub> calculation depends: 0.002 pH from plasma CO<sub>2</sub> content, directly determined; 0.004 from probable variations in the solubility coefficient,  $\alpha$ , of CO<sub>2</sub> in plasma; 0.010 from variations in  $pK'$ ; 0.020 from errors in determining  $p_{CO_2}$  by the method described in this paper. The total is a maximum error of 0.036 pH<sub>s</sub>, which in fact is about that indicated by the data of Table IV, *B*. The maximum total error is to be expected only in the rare case that all the errors from the four values on which the calculation is based are maximal, and in the same direction.

### *Apparatus*

The vessel designed for the equilibration of the blood with a prepared gas mixture is shown in *B*, Fig. 1. The body *B* is of 10 cc. total capacity, the smaller bulb being marked for 1 cc. gas volume, leaving 9 cc. for the blood. Stop-cock *S* is bored at a 90°

angle to allow communication between any two adjacent openings. Stop-cocks and all capillaries leading to them are of 1 mm. bore. The bulb *G*, of 1 cc. capacity, has been introduced so that after blood and gas phase have been brought into equilibrium, the gas may be separated from the blood at 38° before the vessel is removed from the water bath.

#### PROCEDURE

##### *Preparation of Gas Mixtures*

For equilibrating venous blood, a gas mixture having 50 mm.  $p_{\text{CO}_2}$  and 25 mm.  $p_{\text{O}_2}$  at 38° is used. For arterial blood, the initial gas tensions used are 40 mm.  $p_{\text{CO}_2}$  and 80 mm.  $p_{\text{O}_2}$ . The remainder of the gas mixture may be either nitrogen or hydrogen.

The gas mixtures are stored in 300 cc. containers of the type shown as *T* in Fig. 1. We have prepared the gas mixtures with the aid of the gas manifold described by Austin *et al.* (1922, p. 129). The modification introduced by Van Slyke, Wu, and McLean (1923, p. 805), in which the gas mixtures are made up by pressure measurements, has been used. Gas mixtures may thus be rapidly prepared in which the tensions of CO<sub>2</sub> and O<sub>2</sub> are within 1 mm. of those desired.

*Introduction of Gas into Tonometer*—The larger vessel, *T*, in Fig. 1, contains the prepared gas mixture. *B* and the three capillary tubes at its top are filled with mercury. *G* is filled with mercury from *H*. With cock *S* in position 3, a drop of caprylic alcohol is drawn from cup *C* into the capillary below the cup. *C* is then partially filled with mercury from above, and *B* and *T* are connected as shown in Fig. 1. The connecting capillary *X* is of 0.5 mm. bore, and has at its tip a tapered rubber ring, *R*, shown inserted into cup *C*. About 3 cc. of mercury are admitted into *T* from leveling bulb *A*, then capillary *X* is cleared of air by connecting it with *T* and allowing gas from *T* to waste through *X* and bubble out through the mercury in *C*. The interiors of *B* and *T* are connected by turning the proper cocks, then by lowering the leveling bulb *D* the mercury is withdrawn from *B* and replaced by gas from *T*. Stop-cocks *S'*, *S*, and *F* are closed in the order given. *S* is left closed between positions 2 and 3. Clamp *K* is closed and the rubber tube is disconnected from *S'*. Capillary *X* and the mercury are removed from cup *C*.

*Drawing Blood and Introducing It into Tonometer*—The blood is drawn into a tube coated with enough dried neutral potassium oxalate and ammonium fluoride to make the final concentrations in the blood 0.2 and 0.1 per cent, respectively. The necessary volume of a *neutral* solution containing 20 gm. of potassium oxalate

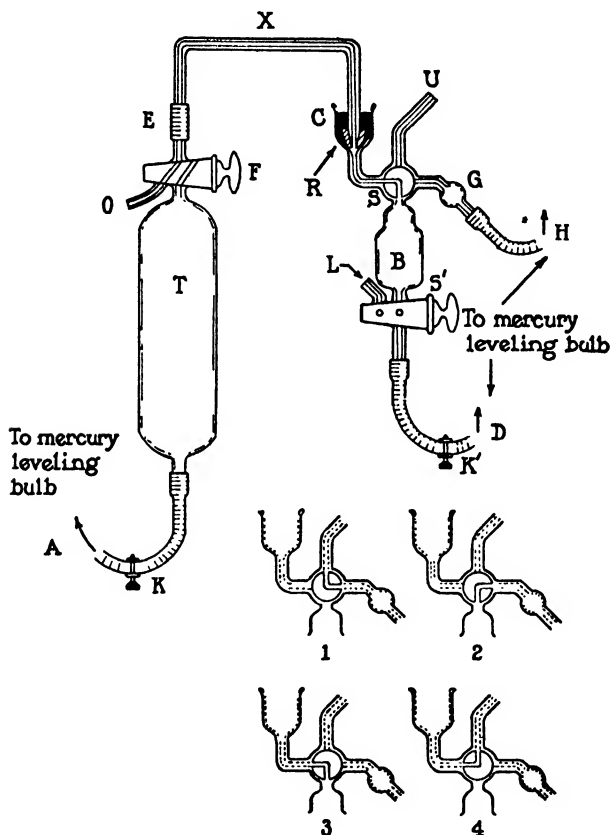


FIG. 1. Apparatus for equilibration of blood. *B* is the tonometer in which the blood is to be equilibrated. *T* is a gas container, from which *B* is about to be filled with a gas mixture approximating the  $\text{CO}_2$  and  $\text{O}_2$  tensions of ordinary venous or arterial blood.

and 10 gm. of ammonium fluoride per 100 cc. is spread on the inside of the vessel and dried with a current of air. The blood is drawn without contact with air. For this purpose it may be

drawn under oil, as described by Van Slyke and Cullen (1917). However, it is preferable to draw it directly into a closed tube over mercury, as described on p. 131 of Austin *et al.* (1922). If first drawn into a tube with oil, the blood is transferred to a closed tube over mercury (tube *J*, Fig. 3, of Austin *et al.*, 1922).

The tonometer, *B*, previously filled with the desired gas mixture, is connected to the blood tube through the lower cock, *S'*. A few drops of blood are run through the outlet *L*. Then stop-cock *S* is opened in position 3 to let out displaced gas, and the vessel is filled through *S'* with blood to the 1 cc. mark. Cock *S* is closed in position midway between positions 3 and 4. Leveling bulb *D* is again connected to the lower stem of tonometer *B*, and the stem is cleared of blood by mercury from *D* wasted through the outlet *L*.

As an alternative procedure, the blood may be drawn into a syringe containing paraffin oil, and forced directly from the syringe into tonometer *B* through a short rubber tube of 2 mm. bore. In this case the necessary layer of oxalate and fluoride is placed on the inner wall of the syringe. During the delivery of blood from the syringe into *B*, the point of the syringe is held downwards, with the connecting tube to *B* bent into a U, so that no oil may enter *B*.

*Equilibration of Blood and Gas at 38°*—The tonometer *B* in an upright position is immersed as far as the upper cock in water at  $38^{\circ} \pm 0.1^{\circ}$ . Leveling bulbs *D* and *H*, attached to the tonometer, are suspended outside the bath. A droplet of mercury is placed in cup *C*. The tonometer is held in the bath for 1 or 2 minutes, then *S* is turned to position 3 to allow escape of enough of the warmed air to lower the pressure within the chamber to atmospheric. The escape of gas is indicated by movement of the droplet of mercury in cup *C*. Cock *S* is closed and the tonometer is left in the bath for another minute, after which *S* is again opened. This procedure is repeated until there is no further indication of the escape of gas when the cock is opened.

Cock *S* is turned from position 3 in a clockwise direction to a position midway between positions 1 and 2. A rubber stopper is inserted into the mouth of cup *C* to keep out water from the bath. The entire tonometer is then immersed in the bath and is rocked in such a manner that the bubble moves from one end of the chamber to the other. An automobile wind-shield wiper, run by

compressed air or vacuum may be used for this purpose. 10 minutes suffice for attainment of  $\text{CO}_2$  equilibrium. At the end of that period the tonometer, still in the bath, is placed in an upright position for 1 or 2 minutes to permit drainage of blood from the wall of the upper part of the chamber. Then, with leveling bulb  $H$  slightly elevated, stop-cock  $S$  is turned to position 2 just long enough to permit a drop-let of mercury from  $G$  to pass into the chamber  $B$ . The mercury removes blood from the bore of cock  $S$ , from which it might otherwise enter  $G$  when the gas is transferred to this bulb.

*Separation of Equilibrated Blood and Gas*—This operation is preferably performed without removing the tonometer from the bath. If one works quickly, however, the tonometer may be taken out and the gas bubble transferred to bulb  $G$  before temperature change has significantly affected the distribution of  $\text{CO}_2$  between the gas and blood.

The tonometer is either removed from the bath, or, preferably, placed in an upright position with only the part above cock  $S$  above the surface of the bath. The stopper is removed from  $C$ . Leveling bulb  $H$  is placed slightly below and leveling bulb  $D$  slightly above the tonometer. Cock  $S$  is then turned to position 2. A portion of the gas from  $B$  escapes at once into  $G$ . Most of the remaining gas is driven into  $G$  by admitting mercury from leveling bulb  $D$  into the bottom of chamber  $B$ . The admission of mercury is stopped when almost all of the gas has been transferred to  $G$ , and before any blood has entered the bore of cock  $S$ . Cock  $S$  is then turned to position 3 and the small bubble of gas left in  $B$ , followed by a little blood, is allowed to escape into cup  $C$ .

$S$  is turned to position 4, and cup  $C$  and the bore of the cock are cleaned by drawing water, and then acetone in succession through  $U$ . The separated gas and blood may now be analyzed at the operator's convenience. If, however, the blood is not analyzed at once, it should be chilled in ice water and kept cold until used. Even then the blood analysis should be made on the same day. Before removal of either the blood or gas for analysis, the tonometer should be brought to room temperature.

*Determination of  $\text{CO}_2$  Content of Gas Bubble*—This analysis is carried out as described in the preceding paper (Van Slyke, Sendroy, and Liu, 1932). The technique described for measuring micro gas samples is followed. To transfer the gas sample from



bulb *G* of Fig. 1 to the Van Slyke-Neill manometric chamber, the arm *U* is connected glass to glass with the side arm of the chamber. Mercury is then run back and forth between cup *C* of the tonometer and the Van Slyke-Neill chamber to drive all gas bubbles out of the connections. Manometer reading  $p_0$  is taken, with the meniscus of the mercury at the 2 cc. mark in the gas-free manometric chamber. The mercury leveling bulb attached to *H* is then placed higher than the leveling bulb of the manometric apparatus, cock *S* is turned to position 1, and all the gas in *G* is passed into the manometric chamber followed by a little mercury to seal the cock of the chamber. The mercury meniscus in the chamber is again brought to the 2 cc. mark and manometer reading  $p_1$  is taken. The pressure exerted by the gas sample at 2 cc. volume is calculated as

$$P_S = p_1 - p_0$$

The absorption of CO<sub>2</sub> with NaOH solution and the rest of the analysis are then carried out as described in the preceding paper (Van Slyke, Sendroy, and Liu, 1932).

*Centrifugation of Blood and Determination of Plasma CO<sub>2</sub> Content*—Tube *X* of Fig. 1 is replaced by another glass capillary, of which the descending outlet limb is long enough to reach to the bottom of a centrifuge tube. The blood is then passed into a centrifuge tube containing a layer of oil. The oil is at once replaced by a layer of low melting paraffin, and the blood is centrifuged. The paraffin is pierced with a warm cork-borer, and 1 cc. samples of the plasma are withdrawn into pipettes and used for determination of the CO<sub>2</sub> content of the blood, according to Van Slyke and Neill (1924).

### Calculation

From the volume per cent CO<sub>2</sub> content of the gas bubble, *C*, the CO<sub>2</sub> tension is calculated by the usual formula.

$$p_{\text{CO}_2} = 0.01 C (B - 49)$$

where *B* is the barometric pressure in mm. of mercury and 49 is the vapor tension of water at 38°.

From the value of  $p_{\text{CO}_2}$  and the CO<sub>2</sub> content of the plasma, the plasma pH is calculated by Equation 1 or 2, previously given. Or

the calculation may be made graphically by the line-chart given in Fig. 1 of Van Slyke and Sendroy (1928), and reproduced in Fig. 87 of Peters and Van Slyke (1931).

*Estimation of Plasma  $\text{CO}_2$  Content from Whole Blood  $\text{CO}_2$  Content and  $\text{CO}_2$  Tension*—The chart in Fig. 2 is analogous to that in Fig.

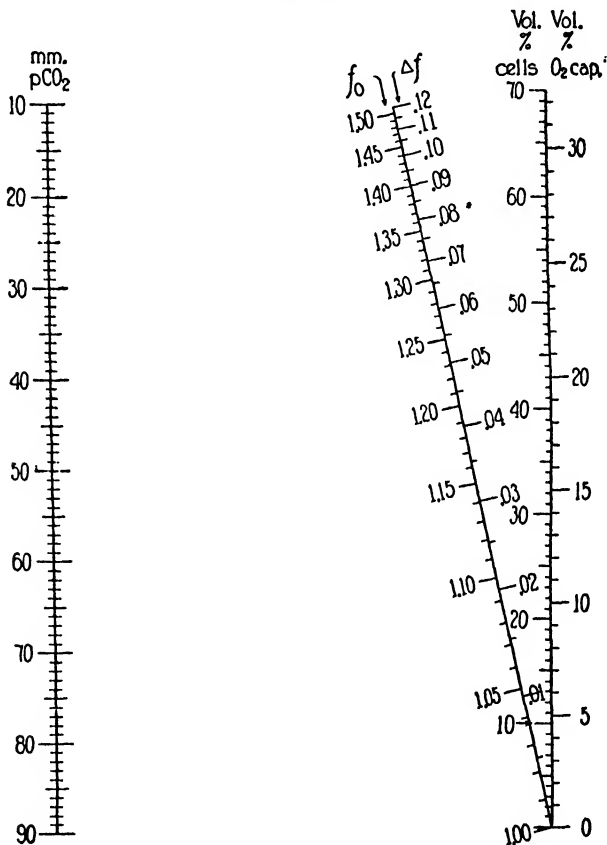


FIG. 2. Line-chart for estimating plasma  $\text{CO}_2$  content from whole blood  $\text{CO}_2$  content.

3 of Van Slyke and Sendroy (1928) for estimating the  $\text{CO}_2$  content of plasma from that of whole blood, with the aid of a factor dependent on the oxygen capacity and  $\text{pH}_s$  of the blood. In the present chart, Fig. 2, a scale for  $\text{pCO}_2$  replaces that for  $\text{pH}_s$ . The

use of the chart is exactly the same as that of the former one, except that  $p_{\text{CO}_2}$  values are used in place of pH. The CO<sub>2</sub> tension values are more convenient in connection with the determinations outlined above, because the  $p_{\text{CO}_2}$  is directly determined. Theoretically, the use of  $p_{\text{CO}_2}$  is not so precise as the use of pH, to correct for the effect of reaction changes on the distribution of bicarbonate, in accordance with Donnan's law, between cells and plasma. In practice, however, we have found little difference between the two charts in the accuracy with which they estimate plasma CO<sub>2</sub> content from whole blood values. The error in plasma CO<sub>2</sub> estimation is usually less than 1 volume per cent, but may be as great as 2.5 volumes per cent.

Fig. 2 can be used to estimate plasma CO<sub>2</sub> contents from whole blood values when working conditions are not convenient for centrifuging the blood to obtain plasma for direct analysis. Any such indirect estimation of plasma CO<sub>2</sub> content, however, increases by about 0.02 the possible error in the pH, calculated, as has been already shown in the discussion of "Calculation of plasma pH by Henderson-Hasselbalch equation, and sources of error involved."

#### EXPERIMENTAL

##### *Blood CO<sub>2</sub> Tensions Set by Saturation with Known Gas Mixtures Compared with Tensions Subsequently Found in Blood by Present Method*

Blood was treated with oxalate and fluoride, as directed for the present method. Then, in order to set the CO<sub>2</sub> and O<sub>2</sub> tensions of the blood at known levels, it was subjected to preliminary saturation with large volumes of gas, in which CO<sub>2</sub> was mixed with H<sub>2</sub>, or with H<sub>2</sub> and O<sub>2</sub>, or with air. The "first saturation method" of Austin *et al.* (1922) was used, in which a tonometer with two chambers is employed, such that, after saturation is finished, the gas phase can be separated for analysis in a large chamber and the blood in a small one. The composition of the gas, with which the blood had been saturated, was determined in a Haldane air analysis apparatus. From the analysis of the gas the CO<sub>2</sub> tension of blood present, given in Tables I to III, was calculated, and, in Table III, also the O<sub>2</sub> tension of blood present. The limit of error of the Haldane air analysis is 0.03 volume per cent of CO<sub>2</sub> or O<sub>2</sub>,

corresponding to 0.2 mm. tension of these gases. The  $\text{CO}_2$  tension of blood present may therefore be considered to be ascertained within 0.2 mm.

Of the blood thus prepared, 9 cc. portions were transferred to tonometer *B* of Fig. 1, and were equilibrated as described in this paper, with 1 cc. portions of analyzed gas mixtures. A 1 cc. bubble was used, consisting of  $\text{CO}_2$  and  $\text{H}_2$  for previously reduced blood

TABLE I

*Determinations of  $\text{CO}_2$  Tension in Reduced Horse and Ox Blood Previously Saturated with Hydrogen Gas Containing Known Tensions of  $\text{CO}_2$*

Blood No.	Initial $\text{CO}_2$ tension of gas bubble	$\text{CO}_2$ tension of blood		
		Present	Found from final $p\text{CO}_2$ of gas bubble	Error
	mm.	mm.	mm.	mm.
1	45.8	41.8	42.5	+0.7
2	45.8	51.6	49.3	-2.3
3	44.1	50.7	51.8	+1.1
4	42.6	38.0	36.3	-1.7
5	42.6	50.2	47.9	-2.3
6	36.8	37.2	37.0	-0.2
7	36.8	44.9	44.3	-0.6
8	44.4	45.6	46.9	+1.3
9	44.4	38.1	38.7	+0.6
10	45.3	50.6	53.0	+2.4
11	45.3	40.8	39.0	-1.8
12	44.7	49.2	48.3	-0.9
13	44.7	40.5	41.5	+1.0
14	45.5	48.6	47.2	-1.4
15	45.5	40.1	41.9	+1.8
16	42.5	35.4	37.1	+1.7
17	42.5	47.6	46.9	-0.7

(Table I), of  $\text{CO}_2$  and air for previously oxygenated blood (Table II), and of  $\text{CO}_2$ ,  $\text{H}_2$ , and  $\text{O}_2$  for blood oxygenated in varying degrees (Table III). The 1 cc. portions of gas used were prepared with  $\text{CO}_2$  tensions, and, in Table III,  $\text{O}_2$  tensions, differing to varying extents from the previously set tensions of the blood, but within such limits of difference as are likely to occur in determinations of the  $\text{CO}_2$  tension of drawn venous or arterial blood.

The results indicate that the error of blood  $\text{CO}_2$  tensions de-

TABLE II

*Determinations of CO<sub>2</sub> Tension in Oxygenated Ox Blood Previously Saturated with Air Containing Known Tensions of CO<sub>2</sub>*

Blood No.	Initial CO <sub>2</sub> tension of gas bubble	CO <sub>2</sub> tension of blood		
		Present	Found from final pCO <sub>2</sub> of gas bubble	Error
	mm.	mm.	mm.	mm.
1	37.6	44.7	43.6	-1.1
2	47.8	43.7	43.0	-0.7
3	44.1	41.6	43.1	+1.5
4	34.2	41.6	41.6	0.0
5	38.4	43.3	43.3	0.0
6	38.4	45.0	45.0	0.0
7	38.4	46.1	44.7	-1.4
8	38.4	36.0	38.3	+2.3
9	43.0	45.9	46.7	+0.8
10	43.0	36.3	36.9	+0.6
11	40.7	45.9	46.5	+0.6
12	40.7	36.3	35.5	-0.8
13	43.6	37.0	38.7	+1.7
14	45.7	37.0	37.7	+0.7
15	40.8	37.0	39.4	+2.4
16	41.7	44.4	44.7	+0.3
17	39.4	44.4	42.4	-2.0
18	43.5	49.1	49.8	+0.7
19	38.5	49.1	50.0	+0.9

TABLE III

*Determinations of CO<sub>2</sub> Tension in Ox, Horse, and Human Blood of Varying Degrees of Oxygenation*

Each blood was previously saturated with a gas mixture containing CO<sub>2</sub>, H<sub>2</sub>, and O<sub>2</sub> at known tensions.

Blood No.	Initial tensions of gas bubble		Gas tensions present in blood		CO <sub>2</sub> tension found in blood	Error in CO <sub>2</sub> tension found
	CO <sub>2</sub>	O <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>		
	mm.	mm.	mm.	mm.	mm.	mm.
1	41.2	140.3	46.6	139.2	46.7	+0.1
2	43.5	52.3	46.6	139.2	43.1	-3.5
3	43.7	73.8	52.3	54.7	51.8	-0.5
4	43.7	73.8	39.9	102.1	39.4	-0.5
5	42.3	68.0	43.9	86.8	45.0	+1.1
6	42.3	68.0	44.3	50.0	43.4	-0.9
7	41.2	66.8	38.9	85.4	38.6	-0.3
8	41.2	66.8	46.8	47.9	46.2	-0.6
9	51.7	30.3	57.5	47.8	57.3	-0.2
10	51.6	41.4	57.5	47.8	59.6	+2.1
11	46.5	61.5	57.5	47.8	59.4	+1.9
12	45.6	70.9	52.8	47.1	51.2	-1.6

terminated as described in this paper averages approximately 1 mm. Except for Blood 2 of Table III, in which the initial oxygen tension of the equilibrating bubble was intentionally made greatly different from that of the blood, the maximum error is 2.4 mm. of  $\text{CO}_2$  tension.

*Comparison of Electrometric and Gasometric pH, Determinations in Human Venous Blood*

The blood was obtained partly from hospital patients and partly from normal individuals. It was collected over mercury without contact with air, as described by Austin *et al.* (1922), in tubes provided with potassium oxalate and fluoride to prevent coagulation and lactic acid formation. No particular effort was made to prevent stasis, hence the  $\text{CO}_2$  tensions are a little higher and the pH, values a little lower than usual for blood drawn from the arm vein without stasis. Each sample of blood was divided into two portions.

Portion I was transferred to tonometer *B* of Fig. 1, and was used for determination of the  $\text{CO}_2$  tension by the method described in this paper. After the gas bubble had been withdrawn from the tonometer for analysis, the residual blood in *B* was used for determinations of blood  $\text{CO}_2$  content, oxygen content, and oxygen capacity by the methods of Van Slyke and Neill (1924). The results of the analyses are given in Table IV, *A*.

Portion II was transferred to a centrifuge tube containing a layer of paraffin oil, which was at once replaced by melted paraffin. The blood was centrifuged under the solidified paraffin. The separated plasma was drawn into a tube over mercury, as described by Austin *et al.* (1922), and was used for electrometric determination of the pH, and, in Bloods 9 to 13, for determination of the plasma  $\text{CO}_2$  content.

For the electrometric pH determination at  $38^\circ$  the electrode chamber of Clark (1915) was used, with thermometer added as described by Cullen (1922). The electrode was filled with about equal volumes of plasma and gas. The gas used was hydrogen to which sufficient  $\text{CO}_2$  was added to give in each case the  $p_{\text{CO}_2}$ , which the blood was found to have by the writers' method in Portion I.

The  $\text{H}_2$ - $\text{CO}_2$  gas mixtures used in the electrometric pH, deter-

TABLE IV  
A. Analyses of Venous Human Blood as Drawn

Blood No.	O <sub>2</sub> capacity of blood	O <sub>2</sub> saturation of blood	CO <sub>2</sub> content of whole blood = [CO <sub>2</sub> ] <sub>b</sub>	CO <sub>2</sub> content of plasma = [CO <sub>2</sub> ] <sub>s</sub>		Determinations of CO <sub>2</sub> tension		
				Determined directly on separated plasma	Estimated from [CO <sub>2</sub> ] <sub>b</sub> by Fig. 2	Initial tensions in gas bubble used in tonometer		Final pCO <sub>2</sub> in gas bubble = pCO <sub>2</sub>
						CO <sub>2</sub>	O <sub>2</sub>	
	vols. per cent	per cent	vols. per cent	vols. per cent	vols. per cent	mm.	mm.	mm.
1	20.93	77.3	57.88		69.27	45.2	67.7	60.4
			57.91		69.72	58.3	67.1	59.4
2	21.55	58.0	55.74		67.05	45.2	67.7	55.1
			55.52		66.65	58.3	67.1	56.5
3	21.48	61.0	53.61		64.10	48.3	67.1	57.2
			53.87		64.70	45.2	67.7	53.6
4	18.46	69.0	55.18		64.98	60.0	44.6	50.2
			55.16		64.95	45.8	67.8	50.0
5	19.77	61.8	54.03		63.93	60.0	44.6	52.0
6	12.47	39.0	61.42		67.45	60.0	44.6	61.8
			61.53		67.60	45.8	67.8	61.9
7	19.78	60.1	57.70		67.92	60.0	44.6	60.2
			58.01		68.30	45.8	67.8	60.7
8	10.68	41.7	60.55		65.52	60.0	44.6	60.9
9	20.77	84.2	54.14	65.62	65.76	44.8	40.2	48.9
10	16.41	55.4	55.08	62.49	62.77	51.6	41.4	55.0
11	21.04	72.3	52.93	63.22	63.78	51.6	41.4	49.5
12	22.42	61.6	53.80	65.04	65.18	46.5	61.5	54.5
13	19.86	65.2	48.00	57.03	56.23	51.7	30.3	57.3
			48.30	57.03	56.50	51.6	41.4	59.6
			48.09	57.03	56.38	46.5	61.5	59.4
14	21.98	42.0	62.00		74.06	46.3	23.7	65.3
			62.00		73.90	49.1	13.8	65.8
15	20.23	67.0	57.14		68.11	46.3	23.7	54.0
			57.14		67.96	47.5	33.2	55.3
16	23.51	80.1	48.82		60.60	40.3	23.4	47.3
			48.93		60.58	48.7	14.7	47.7
			48.87		60.60	48.4	0.0	47.5
17	19.95	50.8	56.76		66.80	46.3	23.4	56.6
			57.17		67.33	48.7	14.7	56.3
			57.19		67.27	48.4	0.0	58.6
18	21.25	31.4	59.17		69.74	46.0	23.5	65.3
			59.36		69.97	48.6	0.0	65.4

TABLE IV—*Concluded*  
*B. pH<sub>s</sub> Values Found in Bloods of Table IV, A*

Blood No.	Gasometric pH <sub>s</sub>		pH <sub>s</sub> by H <sub>2</sub> electrode	Error of gasometric pH <sub>s</sub> if electrometric is exact	
	From pCO <sub>2</sub> and directly determined [CO <sub>2</sub> ] <sub>s</sub>	From pCO <sub>2</sub> and [CO <sub>2</sub> ] <sub>s</sub> estimated from whole blood CO <sub>2</sub>		Gasometric pH <sub>s</sub> from direct [CO <sub>2</sub> ] <sub>s</sub>	Gasometric pH <sub>s</sub> from [CO <sub>2</sub> ] <sub>s</sub> estimated from [CO <sub>2</sub> ] <sub>b</sub>
1		7.30	7.32		-0.02
		7.32	7.32		±0.02
2		7.33	7.35		-0.02
		7.32	7.35		-0.03
3		7.30	7.30		0.00
		7.33	7.30		+0.03
4		7.36	7.39		-0.03
		7.36	7.39		-0.03
5		7.34	7.38		-0.04
6		7.28	7.31		-0.03
		7.28	7.31		-0.03
7		7.30	7.32		-0.02
		7.30	7.32		-0.02
8		7.27	7.29		-0.02
9	7.37	7.38	7.39	-0.02	-0.01
10	7.31	7.30	7.33	-0.02	-0.03
11	7.36	7.36	7.40	-0.04	-0.04
12	7.33	7.33	7.33	±0.00	0.00
13	7.24	7.23	7.20	+0.04	+0.03
	7.22	7.22	7.20	+0.02	+0.02
	7.22	7.22	7.20	+0.02	+0.02
14		7.30	7.32		-0.02
		7.30	7.32		-0.02
15		7.34	7.34		0.00
		7.34	7.34		0.00
16		7.31	7.36		0.00
		7.35	7.36		-0.01
		7.36	7.36		0.00
17		7.32	7.31		+0.01
		7.33	7.31		+0.02
		7.30	7.31		-0.01
18		7.27	7.27		0.00
		7.27	7.27		0.00



minations were made up by pressure as described above for "Preparation of gas mixtures." In this case, however, where exactness was necessary, a correction was required for the change in vapor tension of water between the room temperature, at which the gas mixture was made, and the electrode temperature of 38° to which the plasma and gas were brought for the pH determination. If one represents barometric pressure by  $B$ , room temperature by  $t$ , vapor tension of water at 38° by  $W_{38}$ , and vapor tension of water at  $t^\circ$  by  $W_t$ , the CO<sub>2</sub> pressure that must be measured at room temperature is calculated as follows:

$$\text{Measured } p_{\text{CO}_2} \text{ at } t^\circ = \text{desired } p_{\text{CO}_2} \text{ at } 38^\circ \times \frac{B - W_t}{B - W_{38}}$$

Mixtures made by this method and then subjected to gas analysis were found to be within 1 mm. of the desired CO<sub>2</sub> tension, and usually within 0.5 mm. Any difference in CO<sub>2</sub> tension between plasma and gas put into the electrode chamber would be reduced to less than half by interchange between plasma and gas, so that the final CO<sub>2</sub> tension in the plasma used for electrometric pH estimation can be assumed to be within less than 0.5 mm. of the tension found by our method in blood Portion I. An error of 0.5 mm. in setting the CO<sub>2</sub> tension in the electrode chamber would affect the determined pH by approximately 0.005. It appears probable that the total error of the electrometric pH determination may be considered to be within the limit of  $\pm 0.01$  pH.

The electrodes were standardized with 0.1 N hydrochloric acid, which was assumed to have a pH of 1.08. The system used and the standardization have been discussed, on pp. 708-709 of a previous paper (Van Slyke, Hastings, Murray, and Sendroy, 1925).

The results in Table IV, *B* show a maximum deviation of the gasometric pH, from the electrometric of  $\pm 0.04$  pH. In the majority of cases the deviation does not exceed 0.02 pH.

Since the observed deviations represent the sum of errors between the gasometric and electrometric determinations, it appears that the maximum error in the gasometric pH, does not usually exceed 0.03 pH.

#### SUMMARY

Gasometric methods are described for determining in blood the carbon dioxide tension and the plasma pH.

The CO<sub>2</sub> tension is obtained by equilibrating blood with  $\frac{1}{3}$  its volume of a gas mixture which contains CO<sub>2</sub> and O<sub>2</sub> in tensions approximating those of average venous or arterial blood. The gas bubble attains the CO<sub>2</sub> tension of the blood, which is then determined by micro gas analysis of the bubble with the method described in the preceding paper.

The pH of the plasma is calculated by the Henderson-Hasselbalch equation from the CO<sub>2</sub> tension found in the blood and the CO<sub>2</sub> content determined by analysis of subsequently separated plasma.

The maximum errors are  $\pm 2.5$  mm. of CO<sub>2</sub> tension and  $\pm 0.04$  pH; the usual errors are less.

With one sample of blood and an entirely gasometric technique carried out with the manometric apparatus, these determinations give the acid-base balance of the plasma in terms of pH, CO<sub>2</sub> or bicarbonate content, and CO<sub>2</sub> tension.

The writers are much indebted to Dr. A. Alving and Dr. J. M. Steele for obtaining samples of human blood.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

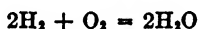
### IV. HYDROGEN AND OXYGEN BY COMBUSTION

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The method here described is an adaptation to the Van Slyke-Neill (1924) manometric apparatus of the familiar principle of hydrogen determination by ignition. The gas is ignited with excess oxygen and the hydrogen is calculated as  $\frac{2}{3}$  of the gas which disappears, in accordance with the reaction:



The method is, of course, applicable without modification only when other combustible gases are absent. An analysis requires about 20 minutes.

For gas analysis by combustion the manometric apparatus, compared with ordinary gas burettes, offers two advantages. (1) Accurate results can be obtained with samples of widely varying size. As little as 1 cc. suffices for analysis with 0.2 volume per cent accuracy, or as much as 30 cc. can be taken, whereby the error is reduced to about 0.05 volume per cent of hydrogen. (2) When the gas mixture is explosive (over 10 per cent hydrogen), it is not necessary to dilute it with air to reduce the explosibility. This object is attained merely by reducing the pressure on the gas.

The same technique serves for oxygen determination when an excess of hydrogen is present. In this case, since combustion diminishes the gas present by 3-fold the amount of oxygen, the analysis can be made with a maximum error of only 0.02 or 0.03 volume per cent of  $\text{O}_2$ .

The general principles of technique and precautions for mano-

metric gas analyses outlined in the introduction to Paper I of this series (Van Slyke and Sendroy, 1932) are to be observed.

### *Apparatus*

Fig. 1 illustrates the construction and manner of attachment of the combustion chamber to the reaction chamber of Van Slyke and Neill. The combustion chamber consists of a heavy walled Pyrex glass tube, of about 100 mm. length and 32 mm. diameter, and therefore about 75 cc. capacity. The mouth of the cylinder

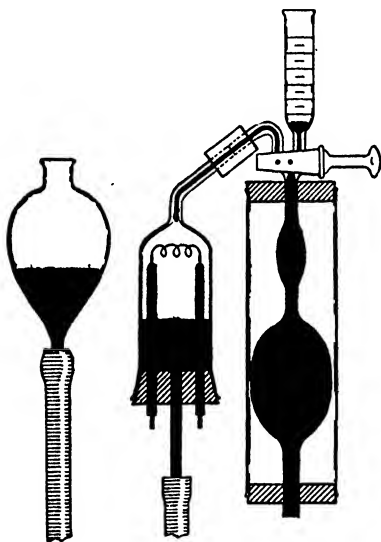


FIG. 1. Manometric and combustion chambers connected. The leveling bulb shown is connected with the combustion chamber.

is expanded slightly so that it will fit the rubber stopper. The glass here is also thickened somewhat and a rim is put on the edge, so that the greased stopper can be forced in without breaking the glass.

Through the stopper pass three glass tubes. One is connected by about 100 cm. of rubber tubing with the mercury leveling bulb. Into the upper ends of the other two are sealed short heavy platinum wires, which are joined within the chamber by a thin platinum wire (No. 26 or 28) about 5 cm. long, bent into three spirals.

After the platinum wires are sealed into the glass, each tube is filled with melted Wood's metal, and a strong copper wire is inserted into the cooling metal. This device, suggested by Dr. Sendroy, enables one to economize on platinum, and to use strong and heavy copper wires for connections below the tubes with the battery wires. Electricity to heat the platinum spiral is provided by a battery of two or three dry cells.

To avoid danger from flying glass in case a miscalculation of the mixture burned should cause a highly explosive one to be ignited (an accident which has not yet occurred), it is well to wrap a layer of wire gauze about the combustion chamber, or slip a mica lamp chimney about it.

The ends of the capillaries connecting the combustion and manometric chambers are ground flat so that they meet with a minimum of dead space. The rubber tube connecting the capillaries from the two chambers is new, heavy walled red "nitrometer" tubing with a small enough bore so that it grips the glass tubes firmly. The same sort of tubing is used to connect the combustion chamber with its leveling bulb.

*In order to insure joints which will not leak under reduced pressure* both the rubber tubing and the rubber stopper used on the combustion chamber are cleaned and boiled with dilute alkali before use, and where rubber fits against glass both surfaces are covered with thin layers of grease before they are joined. The stopper is held tightly in place by wires. It is unnecessary to bind with wire or otherwise the short rubber tube connecting the capillaries of the two chambers. Binding is undesirable because it makes the rubber tube spread slightly, and thereby increases the dead space between the ends of the glass capillaries. The combustion chamber is supported at such a level that its capillary meets that of the Van Slyke-Neill chamber exactly. With these precautions we have never had any perceptible leaks when the pressure in the combustion chamber was reduced to 100 mm. (leveling bulb lowered 660 mm.), which is the maximum evacuation used.

If the leveling bulb of the combustion chamber is lowered so far that a complete Torricellian vacuum is obtained in this chamber, a slight amount of air may enter by diffusion through the thick rubber tube joining the connecting capillaries of the two chambers. To avoid obtaining such a vacuum when evacuating

the combustion chamber, we hang the leveling bulb by a hook from the links of a chain, the lowest link of which does not permit the surface of the mercury in the bulb to fall by more than 660 mm. below the floor of the combustion chamber. (The type of chain used for supporting window sash is convenient.)

The combustion chamber can be easily attached or detached in 1 minute, so that a combustion can form one step in a series of analyses, of which the others are made without the combustion chamber.

In some Van Slyke-Neill manometric chambers the curved outlet capillary approaches so near to the end of the cock that either the bend of the capillary must be changed or the end of the cock ground away to make room for the heavy rubber tube used to attach the combustion chamber.

### *Reagents*

The only reagents required are oxygen and hydrogen, an excess of either being used in determination of the other.

Oxygen can be used in the form of room air for determination of hydrogen, but causes an undesirably great dilution of the sample if the hydrogen content of the latter exceeds 10 per cent. For gas mixtures with higher hydrogen contents pure oxygen is used as the reagent.

Either oxygen or hydrogen can be conveniently introduced into the manometric chamber from a modified Hempel pipette, in the manner illustrated on p. 809 of the paper by Van Slyke and Hiller (1928).

### DETERMINATION OF HYDROGEN IN LARGE SAMPLES, BY PRESSURE MEASUREMENTS WITH THE GAS AT 50 CC. VOLUME

#### *Preparation of Apparatus to Receive Sample*

The combustion chamber is attached and its leveling bulb is routinely located as shown in Fig. 1.

Before the gas sample is admitted a Torricellian vacuum is created in the manometric chamber. Then the connection with the combustion chamber is opened for a second or two. Any air in the connecting capillaries is swept by the rush of mercury into the manometric chamber, from which it is then ejected.

The zero reading,  $p_0$ , is taken with the meniscus of the mercury at the 50 cc. mark, as described by Van Slyke and Sendroy (1932). The gas sample is then admitted as described by the same authors for "Admission of sample estimated by volume," and the  $p_1$  reading is taken as described by them for "Measurement of sample" (p. 518). The pressure  $P_s$  of the sample at 50 cc. volume is measured as  $P_s = p_1 - p_0$ .

#### *Transfer of Sample to Combustion Chamber*

After  $p_1$  has been observed all the gas sample is passed over into the combustion chamber. For the transfer, the leveling bulb of the combustion chamber is lowered a little below the level of the chambers, while the leveling bulb of the manometric chamber is left level with the chambers. The cock at the top of the manometric chamber is opened to connect the two chambers. Mercury is admitted into the bottom of the manometric chamber until all the gas, followed by a little mercury, has been driven over into the combustion chamber. The leveling bulb of the manometric chamber is raised only as much as is necessary to permit the last portions of gas to pass into the combustion chamber. The transfer of gas is accomplished without at any time putting the gas in the manometric chamber under positive pressure, which would be undesirable, since it might cause leakage of some gas out of the cock at the top of the chamber. (The cock is designed to hold against a complete internal vacuum, but cannot be trusted always to hold against positive pressure inside the chamber.)

#### *Addition of Air or Oxygen*

After the sample has been transferred to the combustion chamber enough air or oxygen to burn the hydrogen is measured into the manometric chamber. The desired amount of air or oxygen may be admitted as described by Van Slyke and Sendroy (1932) for admission of samples, either regulated by pressure or regulated by volume. We have generally used the pressure method for regulating the approximate amount of air or oxygen introduced.

Whether air or oxygen shall be used, and how much of either, depends upon the hydrogen content of the sample. The pressure of oxygen present in the mixture burned must exceed half the pressure of the hydrogen. One can always measure an amount of



oxygen into the chamber which will give more than  $0.5 P_s$  mm. of pressure, and be sure that a sufficient excess is present, even if the sample is pure hydrogen.

It is preferable, if the hydrogen content of the sample exceeds 10 per cent, to add pure oxygen rather than air for the combustion. One can then always take a full sized sample, with  $P_s$  of 500 mm. If air is used when the hydrogen exceeds 10 per cent, the size of the sample must be smaller. Otherwise the  $N_2$  of the added air would increase the residual unburned gas, left after explosion, so much that the residual gas could not be measured in one portion.

The minimum amounts of air or oxygen that must be added, for samples of different  $H_2$  and  $O_2$  contents, are indicated by the following formulæ. The formulæ make allowance for any oxygen already in the sample. If the minimum required  $P_{O_2}$  or  $P_{air}$  calculated by these formulæ is zero or negative, it is obvious that enough oxygen is already present in the sample to burn the hydrogen, so that addition of oxygen is unnecessary.

$$(1) \quad \text{Minimum required } P_{O_2} = \left( \frac{\text{per cent } H_2}{200} - \frac{\text{per cent } O_2}{100} \right) \times P_s$$

$$(2) \quad \text{Minimum required } P_{air} = \left( \frac{\text{per cent } H_2}{40} - \frac{\text{per cent } O_2}{20} \right) \times P_s$$

$P_{O_2}$  = pressure of  $O_2$  measured in chamber at 50 cc. volume;  $P_{air}$  = pressure of air similarly measured; per cent  $H_2$  = per cent  $H_2$  in gas sample; per cent  $O_2$  = per cent  $O_2$  in gas sample.

After the desired amount of air or oxygen has been admitted to the manometric chamber the mercury is brought to the 50 cc. mark, and reading  $p_2$  is taken on the manometer.

$$P_A = p_2 - p_0$$

$P_A$  represents the pressure at 50 cc. of the added air or oxygen.

After  $p_2$  has been noted the air or oxygen is transferred to the combustion chamber in the same manner described for transfer of the sample.

### *Combustion*

The manner of combustion depends upon the proportion of hydrogen in the gas mixture burned. If there is less than 10

per cent of hydrogen the mixture will burn quietly at atmospheric pressure without an explosion. If the hydrogen is between 10 and 20 per cent the mixture will burn at atmospheric pressure by explosion, the vigor of which increases with the proportion of hydrogen present. If the hydrogen is less than 20 per cent, however, the explosion is not severe enough to break the glass combustion chamber. The concentration of excess oxygen in the mixture makes no appreciable difference with the vigor of the explosion. A mixture with 9 per cent of hydrogen will burn without explosion, whether the rest of the gas is pure oxygen, or 10 per cent oxygen and 90 per cent nitrogen. And a mixture with 20 per cent of hydrogen explodes with no more apparent violence if the rest of the gas is pure oxygen than if it is nitrogen with only enough oxygen for the combustion.

Even the most explosive mixture, 2 parts of  $H_2$  to 1 of pure  $O_2$ , will burn without explosion if the pressure is so reduced that the partial pressure of hydrogen is below 70 mm. Such reduction of the hydrogen pressure is obtained by reducing the total pressure to 100 mm.

In order to decide the extent to which the ignited mixture must be attenuated to prevent explosion, the hydrogen content of the mixture is calculated from the presumed hydrogen content of the unmixed sample.

$$\text{Per cent } H_2 \text{ in mixture burned} = (\text{per cent } H_2 \text{ in sample}) \times \frac{P_s}{P_s + P_A}$$

*Combustion in One Portion at Atmospheric Pressure*—If the per cent of hydrogen in the gas mixture burned is under 15, one may safely burn the mixture in one portion. After the sample and the added oxygen have been transferred to the combustion chamber, its leveling bulb is put at such a height that the mercury in the chamber is a few mm. above the floor of the chamber, and the wire is heated. If a flash occurs the combustion is complete at once. Otherwise the wire is kept at a red glow for 10 seconds. The current is then shut off, and 30 seconds are allowed for the glass ends, into which the platinum wires are sealed, to cool so that contact with the mercury will not crack them. Then the leveling bulb of the combustion chamber is placed in the position shown in Fig. 1, the leveling bulb of the manometric chamber

somewhat lower, and the cock at the top of the manometric chamber is turned to connect the two chambers. By opening the cock leading to the manometric leveling bulb, the greater part of the gas is drawn over into the manometric chamber. The gas is then returned to the combustion chamber in the manner previously described, in order to wash out any slight pockets or bubbles that may have failed to be transferred to the combustion chamber the first time. With the leveling bulb of the combustion chamber as shown in Fig. 1 the wire is heated again for 10 seconds to burn any traces of hydrogen that may have been washed back from the other chamber.

*Combustion in Instalments under Diminished Pressure*—If the gas mixture contains more than 15 per cent of hydrogen it is burned in two or more instalments under diminished pressure in order to prevent undesirably vigorous explosions. In this case the mixture of gas sample plus air or oxygen, after being mixed in the combustion chamber, is passed back into the manometric chamber, and the cock between the two chambers is closed. If a large sample and much oxygen or air have been taken the total gas present may exceed 50 cc. at atmospheric pressure. In such a case 50 cc. are run back into the manometric chamber, and the rest is left in the combustion chamber. The sample should not be taken so large, however, that this excess is over 10 cc. The leveling bulb of the combustion chamber is lowered to a point 660 mm. below the floor of that chamber. The leveling bulb of the manometric chamber is left level with the bottom of that chamber, and the cock connecting it with the chamber is left open. Then the cock at the top of the manometric chamber is opened to the combustion chamber until enough gas has entered the latter to cause the mercury in it to fall to within a few mm. of the floor of the chamber.

The cock connecting the two chambers is then closed, and the platinum wire is heated for 10 seconds. As the gas burns and contracts the mercury in the combustion chamber rises somewhat. After the current is turned off, at least 15 seconds are allowed for the platinum wire to cool. Then about 10 cc. more of gas are admitted from the manometric chamber, the leveling bulb of the combustion chamber being raised a little, if necessary, to keep the mercury in the chamber above the floor of the latter. The com-

bustion is then repeated as before. This process is repeated until all the gas has been transferred to the combustion chamber and submitted to combustion. The 15 seconds wait after each combustion before the cock connecting the two chambers is reopened is *never to be neglected*, because if the cock is opened while the wire is still warm, even though all glow has ceased, the stream of entering gas striking the wire may ignite, and the flash may strike back into the manometric chamber and produce an undesired explosion.

After the last combustion the gas is once run over into the manometric chamber and back to the combustion chamber, and is exposed to the heated wire again. At this ignition the leveling bulb of the combustion chamber is raised to the level of the chamber, so that the gas is under atmospheric pressure. Complete combustion of final traces of hydrogen appears to be more certain at atmospheric than at diminished pressure.

If the gas mixture contains less than 30 per cent of hydrogen it may be burned in only two portions, the first under  $\frac{1}{2}$  atmosphere pressure, the second under pressure only a little less than atmospheric.

#### *Measurement of Residual Unburned Gas*

After the last heating of the wire, and the 30 seconds interval to permit the glass ends to cool, the unburned gas is returned to the manometric chamber, followed by a little mercury. The mercury meniscus is lowered to the 50 cc. mark, and allowed to rest there 2 minutes while the gas cools. Then  $p_1$  is read. The pressure  $P_R$  of the residual unburned gas is calculated as

$$P_R = p_1 - p_0$$

In case the temperature in the water jacket has changed by more than  $0.1^\circ$  since the initial  $p_0$  reading, the  $p_0$  reading is repeated after the residual gas is ejected from the chamber. The  $p_0$  changes when temperature alters the vapor tension in the chamber, the change being approximately 1.3 mm. with  $1^\circ$  of temperature. The final  $p_0$  reading is the one used to calculate  $P_R$ .

#### *Calculation*

*Temperature Correction to  $P_R$* —If the precautions outlined under "Temperature control" in Paper I of this series (Van Slyke

and Sendroy, 1932) are observed, no significant temperature changes will occur in the chamber between the initial  $p_0$  and the  $p_s$  readings, when the operations are carried through with ordinary rapidity. At the  $p_s$  and final  $p_0$  readings after the combustion, however, the temperature may differ by  $0.2^\circ$  or  $0.3^\circ$  from the initial readings. In such cases a slight correction to the observed  $P_R$  is necessary, in accordance with the gas laws. The correction is made by the formula.

$$P_R \text{ (corrected)} = P_R \text{ (observed)} \times \frac{T_S}{T_R}$$

$T_S$  is the absolute temperature ( $t$  centigrade  $+ 273^\circ$ ) at which  $P_S$  is observed, and  $T_R$  is the absolute temperature at which  $P_R$  is observed. (See p. 513, Van Slyke and Sendroy, 1932.)

*Calculation of Hydrogen*—With the  $P_R$  thus corrected, if necessary, the hydrogen pressure at 50 cc. volume is calculated as:

$$P_{H_2} = \frac{1}{3} (P_S + P_A - P_R)$$

whence

$$\text{Per cent } H_2 = \frac{100 P_{H_2}}{P_S}$$

#### *Analyses by Macro Hydrogen Method*

Table I gives some experimental data and illustrates the method of calculations. The added gas for combustion was pure oxygen in all cases.

To prepare the known gas mixtures several successive portions of nitrogen and hydrogen were measured by pressure in the manometric chamber, in the same manner in which gas samples are measured. The nitrogen and hydrogen so measured were passed into a gas container over mercury, in which they were mixed, and from which samples were withdrawn for analysis.

#### HYDROGEN DETERMINATION IN SMALL SAMPLES, WITH PRESSURE READINGS AT 2 CC. VOLUME

The procedure is similar to, and the calculations identical with, those described above for analyses with pressure measurements at 50 cc. volume. The same combustion chamber attached in the same manner is used.

It is especially important in analysis of the small portions of gas used, to make certain that there are no air pockets in the rubber joint between the manometric and combustion chambers. To remove any gas from this connection before an analysis is begun, the leveling bulbs of both chambers are lowered far enough to give

TABLE I  
*Macro Determination of Hydrogen on Samples of 30 to 35 Cc.*  
Pressure measurements at 50 cc. volume

Analysis No.	Observations								
	$p_0$ (Initial) chamber empty	$p_1$ Sample in chamber	$p_2$ $O_2$ in chamber	$p_3$ Unburned gas in chamber	$p_0$ (Final) chamber empty	Temperature			
	mm.	mm.	mm.	mm.	mm.	Initial °C.	Final °C.		
1	21.8	455.1	610.9*	497.1	22.0	22.8	23.0		
2	21.6	483.1	567.6*	445.9	21.5	22.7	22.6		
3	22.6	591.1	221.0	577.9	22.3	23.6	23.4		
4	22.2	563.2	203.9	544.9	22.1	23.3	23.4		
5	22.0	575.9	133.0	481.7	21.9	23.2	23.3		
6	19.9	565.4	368.7	97.2	20.6	21.0	21.5		
Analysis No.	Calculations								
	$P_S = p_1 - p_0$ (initial)	$P_A = p_2 - p_0$ (initial)	$P_S + P_A$	$P_R$ observed = $p_3 - p_0$ (final)	$P_R$ corrected to initial temperature	Contraction = $P_S + P_A$ - $P_R$	$P_{H_2}$ = t con- traction	$H_2$ found = $\frac{100 P_{H_2}}{P_S}$	$H_2$ present
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	per cent	per cent
1	433.3		589.1*	475.1	474.8	114.3	76.2	17.58	17.56
2	461.5		546.0*	424.4	424.5	121.5	81.0	17.56	17.56
3	568.5	198.4	766.9	555.6	556.0	210.9	140.6	24.72	24.73
4	541.0	181.7	722.7	522.8	522.6	200.1	133.4	24.68	24.73
5	553.9	111.0	664.9	459.8	459.6	205.3	136.9	24.72	24.73
6	545.5	348.8	894.3	76.6	76.5	817.8	545.2	99.95	100.00

\* In Analyses 1 and 2 the oxygen was measured into the manometric chamber without first removing the gas sample. Hence in these two analyses the value  $P_S + P_A$  is observed directly as  $p_2 - p_0$ .

a Torricellian vacuum in the upper part of each. The bulb of the combustion chamber is then raised so that mercury fills that chamber and passes over into the manometric chamber. Any gas bubble previously caught in the connections will be greatly

expanded by this procedure and swept into the manometric chamber, from which it is ejected.

The  $p_0$  reading is determined as in the macro method, except that the meniscus of the mercury is at the 2 cc. mark instead of the 50 cc. one,

The sample is admitted most conveniently from a modified Hempel pipette as described by Van Slyke and Hiller (1928). The sample is admitted with the leveling bulb of the manometric chamber at the medium position, level with the bottom of the chamber, and with the cock open between this bulb and the chamber, so that the gas in the chamber is under slightly less than atmospheric pressure. About 1.5 cc. of sample observed at this pressure is admitted in one portion into the chamber.  $p_1$  is then read with the mercury at the 2 cc. mark in the chamber. It is essential for accuracy that a reading glass be used to observe both the mercury meniscus in the chamber and that in the manometer. As in the macro analysis,  $P_s = p_1 - p_0$ .

The principles governing the measurement of the amount of oxygen or air to add are the same as in the macro analysis. The oxygen or air pressure is measured as  $p_2 - p_0$ , as in the macro analysis, but at 2 cc. volume.

The combustion of the mixed gases is always done at one time, even with the most explosive mixtures. The amount of gas is so small that it does not more than half fill the chamber even when the pressure has been reduced to 100 mm. Even when the mixture does not contain enough hydrogen to be explosive the combustion must be carried out under reduced pressure, merely to expand the gas to sufficient volume to fill the upper part of the combustion chamber, where the wire is.

After the ignition the chamber is allowed to cool for 30 seconds before the mercury is permitted to rise and touch the hot glass tips into which the platinum wire is sealed. The residual gas is then passed into the manometric chamber and back into the combustion chamber, where it is ignited again for 10 seconds. This process is repeated once more, and the gas is ignited a third and final time. The residual gas is then returned to the manometric chamber and its pressure is taken at 2 cc. volume.

The object of the extra passage and ignition is to insure that the last traces of gas which may be trapped in the joint between

the two chambers shall be mixed with the other gases and ignited.

In order to prevent the trapping of any of the residual gas in the joint during the transfer back to the manometric chamber after the final combustion, the leveling bulb of the combustion chamber is left at the low level, 660 mm. below the floor of that chamber,

TABLE II  
*Micro Determination of Hydrogen on Samples of about 1 Cc.*  
Pressure measurements at 2 cc. volume

Analysis No.	Observations								
	$p_0$ (Initial) chamber empty	$p_1$ Sample in chamber	$p_2$ O <sub>2</sub> in chamber	$p_3$ Unburned gas in chamber	Temperature				
					Initial	Final			
	mm.	mm.	mm.	mm.	°C.	°C.			
1	107.0	498.5	586.3*	482.1	22.4	22.5			
2	107.9	538.1	594.9*	483.0	23.2	23.2			
3	107.7	572.1	175.3	467.6	23.6	23.6			
4	107.7	568.0	202.5	490.8	23.5	23.5			
5	107.0	576.3	211.9	506.7	23.1	23.1			
	Calculations								
	$P_S = p_1 - p_0$	$P_A = p_2 - p_0$	$P_S + P_A$	$P_R$ from ob- served $p_2 - p_0$	$P_R$ corrected to initial temperature	Contraction $= \frac{P_S + P_A}{P_R}$	$P_{H_2} = \frac{1}{2}$ con- traction	$H_2$ found = $\frac{100 P_{H_2}}{P_S}$	$H_2$ present
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	per cent	per cent
1	391.5		479.3*	375.1	375.0	104.3	69.5	17.74	17.56
2	430.2		487.0*	375.1	375.1	111.9	74.6	17.33	17.56
3	464.4	67.6	532.0	359.9	359.9	172.1	114.7	24.70	24.73
4	468.3	94.8	555.1	383.1	383.1	172.0	114.7	24.90	24.73
5	469.3	104.9	574.2	399.7	399.7	174.5	116.4	24.78	24.73

\* In Analyses 1 and 2 the oxygen was measured into the manometric chamber without first removing the gas sample. Hence in these two analyses the value  $P_S + P_A$  is observed directly as  $p_2 - p_0$ . In this series  $p_0$  final was assumed to be the same as  $p_0$  initial, since the temperature change during the analysis in no case exceeded 0.1°.

and the manometric chamber is partially evacuated before the cock between the two chambers is reopened. The cock between the manometric chamber and its leveling bulb is then closed, the



two chambers are connected, and the highly rarified gas in the combustion chamber, followed by some mercury, is run into the manometric chamber.

The temperature corrections and method of calculation are identical with those described for the macro analysis. Here, however, the temperature corrections are not so significant because the relative error of the other measurements is greater.

In Table II are given the results of some analyses, and illustrations of the calculations.

#### *Determination of Oxygen*

Either the macro or the micro method above described for the determination of hydrogen serves equally well for determination of oxygen if the combustion is carried out with excess of hydrogen.

Because the contraction on ignition is three times the oxygen volume, the error in oxygen analysis is half that in hydrogen determinations. For oxygen, results are exact within 0.02 or 0.03 volume per cent, about the same as in analyses with the 10 cc. Haldane apparatus.

In three points the procedure for oxygen determination differs from that for hydrogen. (1) For each volume of oxygen present 2 of hydrogen must be added. Hence if the oxygen content of the sample is over 50 per cent the  $P_g$  of the sample must be less than 500 mm. if one is to avoid measuring the hydrogen in two installments. (2) Instead of 10 seconds heating of the wire for each combustion, 30 seconds are needed when the residual gas contains a large proportion of hydrogen. (3) The minimum explosive concentration of oxygen is somewhat less than that of hydrogen. Hydrogen, with an excess of  $O_2$ , is explosive in more than 9 per cent concentration at atmospheric pressure, or when the  $H_2$  partial pressure exceeds 70 mm. Oxygen, with an excess of  $H_2$ , is explosive in more than 7 per cent concentration at atmospheric pressure, or when the  $O_2$  partial pressure exceeds 50 mm.

The procedures for measuring the gas sample, adding the gas required for combustion (hydrogen in this case), carrying out the combustion, and measuring the residual gas, are identical with those described above for determination of hydrogen, except that 30 seconds instead of 10 seconds are given to the ignition.

The amount of hydrogen, measured by its pressure taken at the same volume as  $P_S$ , is estimated as:

$$\text{Minimum required } P_{H_2} = \frac{\text{per cent O}_2 \text{ in sample}}{50} \times P_S$$

For atmospheric air, with 21 per cent oxygen,  $P_{H_2}$  must be at least  $\frac{21}{50} \times P_S = 0.42 P_S$ . The air-hydrogen mixture, to avoid explosions, should be burned in three portions, in macro analyses.

### *Calculations*

The calculations resemble those in the hydrogen determinations, except that here  $P_{H_2}$  replaces  $P_A$  in the hydrogen analyses, and the value of  $P_{O_2}$  is calculated as one-third instead of two-thirds of the contraction.

$$P_{O_2} = \frac{1}{3} (P_S + P_{H_2} - P_R)$$

$$\text{Per cent O}_2 = \frac{100 P_{O_2}}{P_S}$$

### *Determinations of Oxygen in Outdoor Air*

Illustrative results are given in Table III. In these air analyses the amount of hydrogen admitted was such that its pressure was somewhat over 50 per cent of the sample pressure, to insure an excess of hydrogen, of which the required amount is 42 per cent of the air sample.

### *Determination of Both Hydrogen and Oxygen in Same Gas Mixture*

For this purpose the sample is first ignited without addition of either gas. Then the residual gas is mixed with either hydrogen or oxygen, to burn the remainder of whichever of the two gases was in excess in the sample, and a second combustion is performed on the mixture.

### *Combustible Organic Gases*

Methane, acetylene, and other organic gases determinable by combustion can undoubtedly be determined in the same manner as hydrogen, the  $CO_2$  formed being determined when necessary, by

applying gas methods previously described (Van Slyke and Sendroy, 1932; Van Slyke, Sendroy, and Liu, 1932) to the ignited gas mixture.

TABLE III  
*Macro Determinations of Oxygen in Outdoor Air*  
Pressure measurements at 50 cc. volume

Analysis No.	Observations								
	$p_0$ (Initial chamber empty)	$p_1$ Sample in chamber	$p_2$ H <sub>2</sub> in chamber	$p_3$ Unburned gas in chamber	$p_0$ (Final chamber empty)	Temperature			
						Initial	Final		
	mm.	mm.	mm.	mm.	mm.	°C.	°C.		
1	23.4	550.1	292.8	490.3	23.9	24.7	25.0		
2	24.2	567.9	351.3	554.3	24.2	25.3	25.3		
Analysis No.	Calculations								
	$P_S = p_1 - p_0$	$P_{H_2} = p_2 - p_0$	$P_S + P_{H_2}$	$P_R$ from observed $p_1 - p_0$ final	$P_R$ corrected to initial temperature	Contraction = $P_S + P_{H_2} - P_R$	$P_{O_2} = \frac{1}{2}$ contraction	$O_2$ found = $\frac{100 P_{O_2}}{P_S}$	$O_2$ present (assumed for outdoor air)*
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	per cent	per cent
1	526.7	269.4	796.1	466.4	465.9	330.2	190.1	20.90	20.93
2	543.7	327.1	870.8	530.1	530.1	340.7	113.6	20.90	20.93

\* The oxygen content of New York air is sometimes a little below 20.93, because of the amount of coal burned.

#### SUMMARY

Methods are described for determination of hydrogen and oxygen in gas mixtures by the combustion procedure, the gases before and after ignition being measured by pressure in the manometric apparatus of Van Slyke and Neill. The apparatus can be used for micro determinations, with 1.5 cc. samples of gas, or for macro analyses, with samples of 30 to 35 cc. By reducing the pressure in the combustion chamber even the most explosive mixtures can be burned smoothly. They do not need to be diluted with inert gas as in the usual methods. The maximum error in micro determinations is about  $\pm 0.2$  per cent of the total gas. In macro determinations of hydrogen the maximum error is  $\pm 0.05$  per cent, and in oxygen determinations  $\pm 0.03$  per cent of the total gas.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

### V. HYDROGEN BY ABSORPTION WITH PAAL'S PICRATE- PALLADIUM SOLUTION

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The method here described is especially adapted to micro analyses of gas samples of less than 2 cc. volume. It requires about the same time, 20 minutes, as hydrogen determination by the combustion method described in the preceding paper (Van Slyke and Hanke, 1932) and has the same accuracy, about 0.2 volume per cent, as the combustion method with similar small samples of gas. The palladium method has the advantage that it requires no combustion chamber or other accessory apparatus. It has the limitations that it cannot be economically used for more accurate analyses of larger samples of gas, and that preliminary removal of oxygen and carbon dioxide is necessary.

The use of a solution of sodium picrate and colloidal palladium for absorption of hydrogen in gas analysis was introduced by Paal and Hartmann (1910), and has since been used by others (Brunck, 1910; Van Slyke and Binger, 1923).

The general technique for temperature control, admission of samples, etc., discussed in the introductory paper of this series (Van Slyke and Sendroy, 1932) is to be observed in carrying out the operations described below.

#### *General Procedure*

A sample of 1 to 1.5 cc. of the gas mixture, *previously freed from oxygen and carbon dioxide*, is let into the reaction chamber and measured by the pressure exerted at 2 cc. volume. The hydrogen is then absorbed with 0.2 cc. of a palladium-sodium picrate solution.

Because of gradual inactivation of the palladium by the metallic mercury, exposure of the gas to the solution in the chamber is accomplished by a special manipulation to minimize the contact of the palladium with the mercury. After the absorption of the hydrogen is complete, the pressure of the residual gas is read at 2 cc. volume, and the hydrogen is calculated from the decrease in pressure.

The manometric apparatus used is that previously described. (Van Slyke and Neill, 1924; Van Slyke, 1927).

### *Reagents*

*Palladium-Sodium Picrate Solution*<sup>1</sup>—This solution contains 2 per cent of colloidal palladium "nach Paal" (Paal and Hartmann, 1910) and 3.5 per cent picric acid in 0.154 N NaOH. To 3.5 gm. of picric acid, 15.4 cc. of N NaOH and about 50 cc. of water are added, and the mixture is warmed to about 50° until the picric acid is dissolved. Then 2 gm. of colloidal palladium, previously stirred up with about 20 cc. of water, are added, and the whole is made up to 100 cc. It takes about 1 hour with occasional stirring for the palladium solution to become entirely homogeneous.

Since colloidal palladium is gradually inactivated by contact with metallic mercury, it is necessary to avoid introducing mercury into the reagent accidentally. The stock reagent should be kept in a stoppered bottle at a distance from the manometric apparatus in order to avoid contamination with the mercury droplets that are likely to pervade the surroundings. A portion of reagent sufficient for the day's analyses is withdrawn into a small flask.

Paal and Hartmann (1910) used a palladium solution containing 5 per cent picric acid, instead of the 3.5 per cent here described. We have found that from the 5 per cent solution a considerable amount of sodium picrate gradually precipitates on standing, and that by decreasing the concentration to 3.5 per cent, this difficulty can be avoided.

If the picric acid is reduced to triaminophenol by the hydrogen, 0.2 cc. of the reagent combines with 5.5 cc. of hydrogen measured

<sup>1</sup> The colloidal palladium was purchased from Dr. Theodor Schuchardt, Chemische Fabrik, Görlitz, Germany, for \$8.25 a gm. When 0.2 cc. of a 2 per cent solution, or 4 mg., is used, the palladium costs about 3 cents for each analysis.

at 1 atmosphere pressure. The method as described therefore provides theoretically a 4-fold excess of reagent, even when pure hydrogen is analyzed, and in practice has been found adequate for analysis of pure hydrogen.

*Gas-Free Water*—Distilled water is deaerated by the method described by Van Slyke and Neill (1924, p. 534) for the preparation of gas-free reagents. It is then stored in a Hempel pipette over mercury as described by Van Slyke and Hiller (1928, p. 809).

### *Measurement of Gas Sample*

The gas sample is conveniently stored in a modified Hempel pipette described by Van Slyke and Hiller (1928, p. 809). As displacement liquid in the pipette one may use alkaline hyposulfite or pyrogallol solution, which absorbs the  $\text{CO}_2$  and  $\text{O}_2$  from the gas mixture and thereby prepares it for  $\text{H}_2$  determination by this method. The solution in the upper bulb of the Hempel pipette is covered with a layer of oil.

The sample of about 1.5 cc. of gas is admitted into the reaction chamber in the manner described for "Admission of sample regulated by volume" on p. 516 of Paper I of this series (Van Slyke and Sendroy, 1932).

The measurement of the gas sample is carried out as described in Paper I (Van Slyke and Sendroy, 1932),  $p_0$  and  $p_1$  being observed, before and after admission of the gas sample, with the mercury meniscus in the chamber at the 2 cc. mark. The pressure  $P_g$  exerted by the gas sample at 2 cc. volume is calculated as:

$$P_g = c_1 (p_1 - p_0)$$

The correction factor,  $c_1$ , which has a value of about 1.01, is discussed below.

### *Absorption of Hydrogen with Picrate-Palladium Solution*

The leveling bulb of the manometric chamber is placed in its ring level with the bottom of the chamber, and the cock between leveling bulb and chamber is left open, so that the gas in the chamber is under slight negative pressure.

Excess of mercury is removed from the cup of the chamber, so that there is just a little more than necessary to fill the capillary.



0.2 cc. of the palladium-sodium picrate reagent, measured with a pipette to within 0.01 cc., is introduced into the cup. With a thin wire previously dipped into caprylic alcohol, any bubble of air between the palladium solution and the mercury is removed. Now one-fifth, roughly estimated, of the 0.2 cc. of solution is admitted into the reaction chamber. An interval clock is set for a 10 minute period. If the drop of palladium solution admitted remains trapped by capillarity in the top of the reaction chamber, it is necessary to tap the chamber by hand in order to cause the reagent to flow down its walls. After about 10 seconds, when most of the liquid has flown down over the mercury, the stop-cock between the leveling bulb of the apparatus and the gas chamber is closed, and the leveling bulb is lowered to about 76 cm. below the chamber. The cock leading to the leveling bulb is then opened quickly, to let the mercury in the chamber fall 2 or 3 cm. below the 2 cc. mark. The cock is then closed and the bulb is lifted to its ring in the medium position, level with the bottom of the chamber. When the mercury falls the palladium solution is left distributed more or less uniformly over the glass walls of the chamber, and as it gradually drains down after the mercury a large surface of the palladium solution is exposed to the gas phase. After 20 or 30 seconds, when most of the palladium solution has drained down to the mercury surface, the leveling bulb cock is opened slightly, allowing the mercury to rise slowly as far as it will go, pushing almost all of the palladium solution ahead of it. The quick lowering and slow raising of the mercury in the chamber is then once repeated.<sup>2</sup>

The 0.16 cc. of reagent left in the cup is admitted in four instalments. After each addition of reagent the mercury in the chamber is lowered and raised twice in the manner described above. About 2 minutes should be taken for the admission and manipulation of each instalment, so that 10 minutes are taken for complete absorp-

<sup>2</sup> If the mercury were allowed to rise rapidly most of the palladium solution would remain adherent to the glass walls of the chamber. Consequently a large surface of the solution would be exposed to the mercury, and the palladium would be rapidly inactivated. If the mercury rises gradually, in the course of about 10 seconds, practically all of the palladium solution drains upward ahead of the mercury, thus making minimum contact with the mercury surface.

tion of the hydrogen. After the last instalment of solution has been run into the chamber, except the small amount necessary to fill the capillaries of the stop-cock and cup, the cup is half filled with water, and the stop-cock is sealed with mercury.

Admission of 0.04 cc. portions of the palladium solution can, with practice, be managed with sufficient accuracy by means of the well greased cock of the manometric chamber. During the admission the leveling bulb of the chamber is about level with the bottom of the latter, and the cock between chamber and bulb is open, so that the gas in the chamber is under slight negative pressure.

Another procedure, which requires less skill, is the following. The leveling bulb is so held that the mercury surface in it is 2 cm. lower than in the chamber, and the cock between chamber and bulb is then closed, so that the gas in the chamber is under 20 mm. of negative pressure. The cock at the top of the chamber is now opened, and an amount of palladium solution automatically limited to about 0.04 cc. flows into the chamber. This procedure is applicable only with the closed manometer type of apparatus.

#### *Addition of Air-Free Water and Measurement of Residual Gas*

After the hydrogen has been absorbed, 2 to 2.5 cc. of gas-free water are admitted to the chamber. The water dilutes the palladium solution so that it drains more readily, and so that it becomes transparent and its meniscus thus more accurately definable.<sup>3</sup>

The water is admitted into the chamber from a modified Hempel pipette in the manner described by Van Slyke and Hiller (1928, pp. 810 and 811), for admission of CO, except that here the pipette contains water stored over mercury instead of gas over water. It is not necessary to measure the water added more accurately than to keep its volume between 2.0 and 2.5 cc. This volume can usually be estimated with enough accuracy from the space the water occupies in the upper part of the chamber. If the amount

<sup>3</sup> It is not sufficient here to use gas-free water which is stored under oil, nor is it permissible to run the water into the open cup, and from there into the chamber. Even momentary exposure of the water to the atmosphere permits absorption of sufficiently large and variable amounts of air to affect the accuracy of the analysis. It is necessary to store the air-free water over mercury, and to deliver it from its container directly through a mercury seal into the reaction chamber without contact with air.

of water is not thus approximately controlled, a measurable error may be introduced because of different quantities of the residual gas later dissolved by the aqueous phase.

After the water is admitted the Hempel pipette is removed and the stop-cock is sealed with mercury. The mercury meniscus is then lowered to the 50 cc. mark and the chamber is shaken for 2 minutes. Thereby slight amounts of nitrogen gas which have been absorbed by the water during its admission are almost completely returned to the gas phase. (Only about 0.001 of the total  $N_2$  in the chamber remains dissolved under the conditions of equilibration.) The water meniscus is then brought to the 2 cc. mark and the manometer reading  $p_2$  is taken. The temperature is recorded.

The gases are ejected without loss of liquid as described in a previous paper (Van Slyke, 1926-27, p. 240). The stop-cock is sealed with mercury, the solution meniscus is brought exactly to the 2 cc. mark, and the manometer reading,  $p_3$ , is taken. Expressing the pressure of the residual gas as  $P_R$ , we have

$$P_R = p_2 - p_1 - c_2$$

The  $p_3$  reading ends the analysis.

#### *Determination of $c_1$ Correction*

The fact that the difference in curvature of menisci makes the volume of gas over a mercury meniscus at the 2 cc. mark in the chamber exceed by about 1 per cent the volume over a water meniscus at the same mark, has been discussed by Van Slyke and Sendroy (1932). Since  $P_S$  is measured with the gas over mercury and  $P_R$  with it over water, a correction must be made to  $P_R$  for the above mentioned volume difference in order to make  $P_R$  accurately comparable with  $P_S$ . There is also a slight additional correction to  $P_R$  required for the slight amount of  $N_2$  that is dissolved by the 2.2 to 2.7 cc. of water in the chamber. After this water has been shaken *in vacuo* as directed for the last stage of the analysis, about 0.001 of the  $N_2$  in the chamber remains in solution. And when the gas volume is diminished again to 2 cc. a little more  $N_2$  dissolves, so that the total dissolved is about 0.002 of that present.

The correction,  $c_1$ , for the combined effects of the difference between mercury and water menisci, and for dissolved  $N_2$ , is

determined as follows: A reading,  $p_0$ , is taken with no gas or visible water in the chamber, and with the mercury meniscus at the 2 cc. mark. Then enough air is admitted to exert 400 to 500 mm. pressure at 2 cc. volume, and  $p_1$  reading is taken, again over mercury. 2 cc. of air-free water are now admitted to the chamber, the mercury is lowered to the 50 cc. mark, and the chamber is shaken for 2 minutes. The water meniscus is now brought to the 2 cc. mark and the reading  $p_2$  is taken. Finally the air is ejected from the chamber, without loss of more than 0.1 cc. of the water, and the reading  $p_3$  is taken with the chamber gas-free and the water meniscus again at the 2 cc. mark. Then

$$c_1 = \frac{p_2 - p_3}{p_1 - p_0} = \frac{\text{pressure over water}}{\text{pressure over mercury}} = \text{about 1.01}$$

The exact value of  $c_1$  is determined by repeating several times the above determination and taking the average result. The value

TABLE I  
*Determination of  $c_1$  for Authors' Apparatus*  
Pressure readings at 2 cc. volume

Analysis No.	$p_0$	$p_1$	$p_2$	$p_3$	$c_1 = \frac{p_2 - p_3}{p_1 - p_0}$	Temperature at $p_1$ reading	Temperature at $p_3$ reading
	mm.	mm.	mm.	mm.		°C.	°C.
1	105.0	517.3	515.2	99.0	1.0095	20.9	21.0
2	109.2	545.0	525.5	86.1	1.0082	24.9	25.0
3	106.8	536.4	519.4	85.6	1.0097	22.4	22.5

of  $c_1$  can be thus determined with a precision of about 1 part per 1000 (see Table I).

*Correction,  $c_2$ , for Air Carried into Chamber Dissolved in 0.2 Cc. of Palladium Solution*—The palladium solution when admitted is saturated with air, which it gives off in the chamber. To determine  $c_2$ , 1 cc. of the palladium solution and 1 cc. of completely air-free water are measured into the chamber. The mercury is lowered to the 50 cc. mark and the solution is extracted by shaking for 2 minutes. The pressure,  $p_1$ , is then measured at 2 cc. volume. The gas is ejected, and  $p_2$  is read.

$$c_2 = 0.2 (p_1 - p_2)$$

In this case 5 times the usual amount of palladium solution is taken and the pressure of the extracted air is divided by 5 to obtain the correction for the amount of reagent used in the analyses. The  $c_2$  correction is usually about 1.5 mm.

*Correction for Temperature Changes*—If the temperature in the water jacket of the chamber changes during the interval of 10 or 12

TABLE II

*Analyses of Hydrogen-Nitrogen Mixture Containing 17.56 Per Cent Hydrogen*  
Pressure readings at 2 cc. volume

Analysis No.	Observations					
	$p_0$ Chamber empty	$p_1$ Sample in chamber	$p_2$ After $H_2$ absorption	$p_3$ Reagent solution in chamber	Temperature at $p_1$ reading	Temperature at $p_3$ reading
	mm.	mm.	mm.	mm.	°C.	°C.
1	107.0	543.8	449.2	84.3	22.7	22.7
2	112.0	548.3	451.3	86.9	23.5	23.6
3	108.0	573.0	474.1	85.9	23.6	23.9
4	107.7	500.3	413.2	85.0	23.1	23.3
Analysis No.	Calculations					
	$P_S$		$P_R$		$P_{H_2} = P_S - P_R$	$H_2$
	Uncorrected, $p_1 - p_0$	Corrected by $c_1$ of 1.009	Uncorrected, $p_2 - p_1$	Corrected by $c_2$ of 1.5 mm.		
	mm.	mm.	mm.	mm.	mm.	per cent
1	436.8	440.7	364.9	363.4	77.3	17.54
2	436.3	440.2	364.4	362.9	77.3	17.54
3*	465.0	469.2	388.2	386.3	82.9	17.65
4	392.6	396.1	328.2	326.7	69.4	17.52

\* In Analysis 3,  $\frac{1}{1000}$  of  $p_2 - p_1$  is deducted for temperature correction. The temperature changes in the other analyses are not significant.

minutes between the  $P_S$  and  $P_R$  readings, the  $P_R$  reading is multiplied by the factor,  $\frac{T_S}{T_R}$ , in which  $T_S$  and  $T_R$  represent the absolute temperatures at the readings of  $P_S$  and  $P_R$ , respectively. (See Equation 1 and discussion of temperature corrections in Paper I of this series (Van Slyke and Sendroy, 1932, p. 513).)

*Calculation**A. Hydrogen in CO<sub>2</sub>- and O<sub>2</sub>-Free Gas Analyzed*

$$P_{H_2} = P_S - P_R$$

$$\text{Per cent H}_2 = 100 \times \frac{P_{H_2}}{P_S}$$

*B. Hydrogen in Original Gas Containing CO<sub>2</sub> and O<sub>2</sub>*

$$\text{Per cent H}_2 = A \times \frac{100}{100 - (\text{CO}_2 + \text{O}_2)}$$

$A$  = per cent of H<sub>2</sub> calculated by Formula A;  $(\text{CO}_2 + \text{O}_2)$  = per cent of CO<sub>2</sub> + O<sub>2</sub> in original gas mixture. The value of (CO<sub>2</sub> + O<sub>2</sub>) must be determined by separate analysis if the H<sub>2</sub> content of the original gas mixture is desired.

*Analyses of Known Mixtures of Hydrogen and Nitrogen*

Known mixtures of the two gases were prepared by running successive portions of about 30 cc. of the gases into the chamber of

TABLE III

*Analyses of Hydrogen-Nitrogen Mixture Containing 49.88 Per Cent Hydrogen*  
Pressure readings at 2 cc. volume

Observations							
Analysis No.	$p_0$ Chamber empty	$p_1$ Sample in chamber	$p_2$ After $H_2$ absorption	$p_3$ Reagent solution in chamber	Temperature at $p_1$ reading	Temperature at $p_2$ reading	
	mm.	mm.	mm.	mm.	°C.	°C.	
1	103.3	482.1	280.0	85.1	20.6	21.3	
2	105.4	482.5	280.1	86.8	22.5	22.5	
Calculations							
Analysis No.	$P_S$		$P_R$			$P_{H_2} = P_S - P_R$	$H_2$
	Uncorrected, $p_1 - p_0$	Corrected by $c_1$ of 1.009	Uncorrected, $p_2 - p_1$	Corrected by $c_2$ of 2.6 mm.	Corrected to temperature of $P_S$		
	mm.	mm.	mm.	mm.	mm.	mm.	per cent
1	378.8	382.2	194.9	192.3	191.9	190.3	49.78
2	377.1	380.5	193.3	190.7	190.7	189.8	49.90

the manometric apparatus, measuring the pressures at 50 cc. volume, and transferring the portions to gas containers over mercury. In Tables II to IV are given the analyses of two mixtures so prepared and of a sample of commercial hydrogen.

*Absorption of Hydrogen in Presence of Oxygen*

The presence of oxygen in amounts equivalent to the hydrogen (1 part of oxygen to 2 parts of hydrogen) markedly decreases the

TABLE IV  
*Analyses of Commercial Hydrogen*  
Pressure readings at 2 cc. volume

Analysis No.	Observations					
	$p_0$	$p_1$	$p_2$	$p_3$	Temperature at $p_1$ reading	Temperature at $p_3$ reading
	mm.	mm.	mm.	mm.	°C.	°C.
1	107.5	512.0	8.91	8.75	24.5	24.5
2	104.8	509.0	8.69	8.53	22.0	22.0
3	105.2	431.8	8.71	8.60	22.6	22.6
Analysis No.	Calculations					
	$P_S$		$P_R$		$P_{H_2} = P_S - P_R$	H <sub>2</sub>
	Uncorrected, $p_1 - p_0$	Corrected by $c_1$ of 1.009	Uncorrected, $p_3 - p_2$	Corrected by $c_3$ of 1.5 mm.		
	mm.	mm.	mm.	mm.	mm.	per cent
1	404.5	408.1	1.6	0.1	408.0	99.98
2	404.2	408.8	1.6	0.1	408.7	99.98
3	328.6	329.5	1.1	-0.4	329.9	100.12

rate of hydrogen absorption by the reagent, and makes the absorption very incomplete in the 10 minute period above prescribed. Larger amounts of oxygen further decrease the rate of hydrogen absorption. On the other hand, in the presence of a great excess of hydrogen, small quantities of oxygen are partially absorbed along with all of the hydrogen by the above described procedure. This inconstant behavior of oxygen makes it necessary to remove oxygen before attempting the absorption of hydrogen by the methods described. Table V gives the results of analyses in the presence of oxygen.

CO<sub>2</sub> must also be removed before the analysis, because it would be absorbed by the alkaline palladium solution.

TABLE V

*Results of Attempts to Determine Hydrogen by Absorption with Picrate-Palladium Solution in Presence of Oxygen*

Analysis No.	Composition of gas mixtures used			Result of H <sub>2</sub> analysis	$\frac{\text{H}_2 \text{ found}}{\text{H}_2 \text{ present}}$
	Hydrogen	Oxygen	Nitrogen		
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	17.56	0.00	82.44	17.56	100.00
2	15.70	10.56	73.74	6.62	42.15
3	100.0	0.00	0.00	99.88	99.88
4	86.05	13.95	0.00	99.58	115.6*
5	67.21	32.79	0.00	49.10	73.02
6	63.27	36.73	0.00	36.79	58.14

\* Greater than 100 because of absorption of some oxygen along with all of the hydrogen.

## SUMMARY

A method is described for the quantitative estimation of hydrogen in gas mixtures by absorption with a colloidal palladium-sodium picrate solution in the manometric gas apparatus of Van Slyke and Neill. A complete analysis can be performed in 20 minutes. With samples of 1.5 cc. the maximum error is 0.2 per cent of the total gas.

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## MANOMETRIC ANALYSIS OF GAS MIXTURES

### VI. CARBON MONOXIDE BY ABSORPTION WITH BLOOD

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For determination in air of small amounts of CO, such as are encountered in physiological experiments or as atmospheric contaminations, a principle employed by Arnold, Carrier, Smith, and Whipple (1), and by others (2-5) is, with certain refinements, again applied in this paper. The O<sub>2</sub> is removed, and from the N<sub>2</sub>-CO residue the CO is absorbed by reduced blood. The absorbed CO in the blood is then measured by the technique of blood gas analysis. The latter is carried out, with slight modification, according to the method of Sendroy and Liu (6) for the determination of CO in blood. In the absence of oxygen, the affinity of reduced ox blood for carbon monoxide is so great that, even when the concentration of CO in the gas is only 0.05 per cent, it is absorbed by the blood, under the conditions of this analysis, to the extent of 93.7 + 2.4 per cent.

Carbon monoxide in air, in concentrations of from 0.05 to 0.3 volume per cent, can be determined with a maximum error of  $\pm 5$  per cent of the amount present. The CO estimation can conveniently be combined with determination of oxygen by the method of Van Slyke and Sendroy (7), in the same air sample.

#### *Reagents*

With the exception of the air-free 1 N NaOH, which is omitted here, the reagents required are those used by Sendroy and Liu ((6) p. 134). However, it has been found advantageous to increase the volume of concentrated lactic acid from 8 to 10 cc. per liter in the preparation of the acid ferricyanide reagent. In addition, the following solutions are prepared.

(a) *Sodium hyposulfite, 20 per cent solution with anthrahydro-*

*quinone sulfonate catalyst*, for absorption of  $O_2$  from air, the preparation of which is described by Van Slyke ((8) p. 124). This  $Na_2S_2O_4$  solution, according to Sendróy (9) may most conveniently be kept over mercury.

(b) *Sodium hyposulfite, 20 per cent solution without catalyst*, for blood reduction, is also prepared as above, 10 cc. at a time, and is kept under oil for the day's work.

(c) *Fresh ox blood* is reduced as described below.

### Procedure

*Measurement and Deoxygenation of Gas Sample*—The cleaned chamber of the manometric apparatus of Van Slyke and Neill (10) is connected through the capillary side arm at the top with a gas sample container of about 40 or 50 cc. capacity, with leveling bulb attached, as shown in Fig. 1 of Van Slyke, Sendroy, and Liu (11). The container is filled with mercury, as are the connecting tubes, so that no gas remains in the system. For the connection, a rubber tube with heavy walls and small bore is used. The tube should be just long enough to permit shaking of the chamber without inconvenient tugging on the gas container.

From another container the sample of gas, about 35 cc., is admitted into the chamber through a mercury seal in the cup, as shown in Fig. 1 of Van Slyke and Sendroy (7). The manometric readings,  $p_0$  and  $p_1$ , are taken as described by Van Slyke and Sendroy, before and after admission of the gas sample, and the sample is measured by its pressure,  $P_s$ , at 50 cc. volume.

$$P_s = p_1 - p_0$$

The sample usually taken for this work is such that  $P_s$  is about 500 mm. at room temperature.

To remove oxygen (and  $CO_2$ ) from the sample, the latter is shaken with 3.0 cc. of alkaline hyposulfite solution as described on p. 526 of Van Slyke and Sendroy (7) for determination of  $N_2$  in air.<sup>1</sup> The oxygen-free gas in the chamber is then run through the

<sup>1</sup> If the  $O_2 + CO_2$  content of the gas sample is desired, a reading  $p_1'$  is taken immediately after the deoxygenation of the gas sample, with the mercury meniscus at the 50 cc. mark in the chamber, the 3 cc. of hyposulfite solution over the mercury, and the unabsorbed  $N_2 + CO$  in the 47 cc. gas space.

chamber's capillary outlet into the mercury-filled container already attached. The hyposulfite is permitted to follow the gas into the narrow upper part of the chamber until the solution *just* touches the stop-cock.<sup>2</sup> The stop-cock is then turned, the hyposulfite is ejected into the cup above the chamber, and the chamber is cleaned with water. The deoxygenated gas sample is kept in the attached container under *slightly* positive pressure, until ready for equilibration with the reduced blood.

*Removal of Air from Ferricyanide Reagent*—1 drop of caprylic alcohol and 15 cc. of the acid ferricyanide reagent are run into the chamber of the manometric apparatus and freed of air. The dissolved air is removed by evacuating the chamber, shaking 3 minutes, ejecting the extracted air, and repeating the process once more. The gas-free reagent is then forced up into a 25 cc. burette in the manner described by Van Slyke and Neill ((10) p. 535) for the handling and storage of air-free reagents under oil. The burette should contain sufficient oil to make a layer of at least 10 cm. deep, and the tip, which must extend long enough to reach the bottom of the chamber cup, is provided with a rubber ring to make a tight connection. The burette is set aside until the reduced blood has been prepared for the analysis. A fresh portion of reagent must be freed of air for each analysis, because the layer of oil prevents reabsorption of significant amounts of air for only about a half hour.

*Preparation of Reduced Blood*—The manometric chamber is washed, and a 5 cc. portion ( $\pm 0.1$  cc.) of ox blood, of normal hemoglobin content, is run into the cleaned chamber. 2 drops of caprylic alcohol are added, and the chamber is evacuated and shaken for 3 minutes. The extracted gases are ejected without loss of solution as described by Van Slyke ( (12) p. 240). 1 drop of the sodium hyposulfite solution *without anthrahydroquinone sulfonate catalyst* is added to the blood and the extraction *in vacuo* is then repeated, this time for 2 minutes. After this treatment, there is no measurable amount of oxygen left in the blood. After

<sup>2</sup> When analyzing for  $O_2 + CO$ , the stop-cock is turned at this point, a little of the hyposulfite solution is run up into the capillary leading to the cup of the chamber, the stop-cock is sealed with mercury, and a reading  $p_{O'}$  is taken with the *mercury* meniscus at the 50 cc. mark. The hyposulfite is then ejected.

each extraction the ejection of the gas from the chamber must be complete. If a drop of blood follows the ejected bubble of gas up into the cup its loss is of no importance. The drop of blood which is exposed to air in the cup is not returned to the chamber, but is removed from the cup before the stop-cock is sealed with mercury for the next extraction.

*Absorption of Carbon Monoxide by Reduced Blood*—The de-oxygenated gas from the attached container is admitted into the chamber over the reduced blood. It is followed by a little mercury, to fill the connecting tubes, and to seal the stop-cock of the chamber. After this the attached container is disconnected.

The leveling bulb of the chamber is placed in a position slightly lower than the bottom of the chamber, and the stop-cock between the two is opened. *The water jacket of the manometric chamber is completely covered with tin foil to exclude all light* (the tin foil may be fastened in place with adhesive tape). The chamber is then slowly shaken for 30 minutes. The speed of shaking should be such that the blood, floating on the 10 or 15 cc. of mercury in the chamber, is whirled about the wall but is not broken up into foam. After equilibration, the tin foil is removed and the unabsorbed gas is ejected without any loss of blood. 4 drops of caprylic alcohol are added to the reduced blood in the chamber, and the stop-cock of the chamber is sealed with mercury.

*Determination of CO Absorbed by Blood*—Through a mercury seal ((8) p. 126) 13 cc. of the air-free ferricyanide reagent from the oil-protected burette are admitted into the chamber with the blood. Before the acid ferricyanide is admitted the chamber is partly evacuated, so that the surface of the blood is in the broad part of the chamber. After each 2 cc. admitted, the stop-cock of the chamber is closed, and while the burette is held firmly in place, the chamber is shaken gently two or three times to avoid the formation of large masses of protein precipitate which would be likely to adhere to the glass walls.

After the addition of the ferricyanide reagent, the stop-cock of the chamber is sealed with mercury, and the mercury in the chamber is lowered to or slightly below the 50 cc. mark, so that when the chamber is shaken mercury will not be thrown about its walls, with reduction of the ferricyanide. The evacuated chamber is shaken for 7 minutes to extract the CO from the large volume of solution.

From this point, the procedure for the determination of the CO absorbed by the blood is that described by Sendroy and Liu. The following additions to the technique are matters of convenience.

Before the Hempel pipette is used, some of the pyrogallate solution, together with any blood coagulum which may be present from a preceding analysis is run out to clear the stop-cock *a* (Fig. 1 of Sendroy and Liu (6)) of any air that may be present. Then a little caprylic alcohol is put into cup *c* and a drop is admitted into the capillary *r*. The caprylic alcohol prevents foam formation in the pyrogallate, and increases the ease with which the bubble of  $N_2 + CO$  gas can be returned to the chamber. In using the Hempel pipette, it is convenient to support its weight by a light wire or chain.

After removal of the blood solution from the chamber, the latter may be easily cleaned by the use of small portions of alcoholic KOH and alkaline hyposulfite. When the gas is returned to the chamber from the Hempel pipette, it is followed by the drop of caprylic alcohol. During the absorption of CO by cuprous chloride, some of the absorbent may precipitate on the walls of the chamber. After the analysis is finished the precipitate is readily dissolved by a mixture of saturated NaCl and 1 N  $H_2SO_4$ .

### Calculation

The pressure at 50 cc. of the sample, is calculated as

$$(1) \quad P_S = p_1 - p_0$$

The CO pressure at 0.5 cc. volume is calculated as

$$(2) \quad P_{CO} = p_2 - p_3 - c$$

$$(3) \quad \text{Per cent CO} = \frac{f_1 P_{CO}}{f_1 P_S} \\ = \frac{100 \times \text{cc. CO in sample}}{\text{cc. volume of sample}}$$

$p_2$  and  $p_3$  are the readings taken before and after the absorption of CO with Winkler's cuprous chloride solution in the Sendroy-Liu blood analysis.

$f_1$  is the factor from Table I, corresponding to the temperature and volume (0.5 cc.) at which  $P_{CO}$  is determined.

TABLE I

*Factor  $f_1$  by Which  $P_{CO}$ , Measured at 0.5 Cc. Mark, Is Multiplied to Give 100 Times the Volume, in Cc. at 0°, 760 Mm., of Carbon Monoxide Present in Gas Sample Analyzed*

Temperature	Factor $f_1$
°C.	
10	0.0687
11	85
12	82
13	79
14	77
15	74
16	71
17	69
18	67
19	64
20	62
21	60
22	57
23	55
24	52
25	49
26	47
27	45
28	43
29	40
30	38
31	35
32	33
33	31
34	29

$f_2$  is the factor from Table II, corresponding to the temperature and volume (50 cc.) at which  $P_g$  is determined.<sup>3</sup>

<sup>3</sup> The calculation for the amount of  $O_2 + CO_2$  present, when the readings  $p_1'$  and  $p_0'$  are taken,<sup>1,2</sup> is performed as follows: From the  $p_1'$  and  $p_0'$  readings taken with the gas at 47 cc. volume, the pressure at 50 cc., of the  $N_2 + CO$  left after hyposulfite treatment is given by the equation

$$(4) \quad P_{N_2 + CO} = 0.94 (p_1' - p_0')$$

(See discussion of calculation by Van Slyke and Sendroy (7).) The content

TABLE II

*Factor  $f_2$  by Which  $P_s$ , Measured at 50 Cc. Mark, Is Multiplied to Calculate the Volume in Cc. of Gas Sample Reduced to 0°, 760 Mm.*

Temperature	Factor $f_2$
°C.	
10	0.0634
11	31
12	29
13	27
14	24
15	, 22
16	20
17	18
18	15
19	13
20	11
21	09
22	07
23	05
24	02
25	00
26	0.0598
27	96
28	94
29	92
30	90
31	88
32	86
33	84
34	82

of  $N_2 + CO$  is given by the equation

$$(5) \quad \text{Per cent } N_2 + CO = \frac{100 P_{N_2 + CO}}{P_s}$$

from which the oxygen, and the  $CO_2$ , which is negligible in ordinary atmospheric air, are calculated as

$$(6) \quad \text{Per cent } O_2 + CO_2 = 100 - \text{per cent } (N_2 + CO)$$

In atmospheric air the  $CO_2$  is usually negligible in comparison with the  $O_2$ , so that Equation 6 gives the  $O_2$  content.



The factors of Table I are calculated by Equation 4 of Van Slyke and Neill (10), which gives the cc. of CO reduced to 0°, 760 mm., in the blood sample analyzed. The factors thus obtained are multiplied by 1.067 to give the cc. of CO in the gas sample, since only 0.937 or  $\frac{1}{1.067}$  of the CO in the gas is absorbed by the blood. The factors obtained are then multiplied by 100, in order to give at once results in volumes per cent. The complete calculation of the factors is therefore expressed by the equation:

$$\text{Factor } (f_1) = \frac{a}{760 (1 + 0.00384 t)} \left( 1 + \frac{S}{A - S} \alpha' \right) \times 106.7$$

$t$  is the temperature in degrees centigrade;  $a$  is the volume, 0.5 cc., at which the pressure  $P_{\text{CO}}$  is measured;  $S$  is the volume of solution, 18 cc., present in the chamber when the CO is extracted from the blood-ferricyanide mixture;  $A$  is the capacity of the chamber, 50 cc.;  $\alpha'$  is the distribution coefficient of CO between gas and water phases, as shown in Table I of Van Slyke and Neill.

The factors of Table II are calculated from the equation:

$$\text{Factor } (f_2) = \frac{a}{760 (1 + 0.00384 t)} = \frac{0.0658}{1 + 0.00384 t}$$

when  $a = 50$  cc.

The  $c$  correction is determined by repeating the procedure described above in all respects, except that the steps involved in handling the air sample are omitted. The blood is reduced, the air-free ferricyanide reagent is at once added, and the determination from this point is finished as above. Two readings are made at the 0.5 cc. mark,  $p_1$  and  $p_2$  (corresponding to readings  $p_2$  and  $p_3$  of the air analysis) before and after the addition of Winkler's solution to absorb CO. The  $c$  correction is calculated as

$$p_1 - p_2 = c$$

The  $c$  correction is the sum of two components. One is the  $c_2$  correction of the Sendroy-Liu method ((6) p. 139). The other is a correction for a slight amount of CO (0.08 to 0.18 volume per cent) which, in agreement with the work of Nicloux (3) and

McIntosh (4), has been found to be apparently present in normal blood. Because this CO content of the blood is variable, the *correction must be determined with a reduced portion of the same blood that is used for the air analysis.*

**Correction Factor 1.067**—The reaction,  $\text{Hb} + \text{CO} \rightleftharpoons \text{HbCO}$  is a reversible one. Since the final result of shaking the  $\text{N}_2 + \text{CO}$  mixture with the blood must be an equilibrium between the CO in the gas phase and the CO bound to hemoglobin in the liquid phase, it is theoretically impossible that all of the CO present should be combined with hemoglobin. There is not much known about the system: reduced hemoglobin, CO hemoglobin, and CO (13). However, since there is a variation in the blood of different species, in the oxygen dissociation curves, and in the relative affinity constant of hemoglobin with respect to  $\text{O}_2$  and CO, it is not unreasonable to expect that there may also be a similar species specificity with respect to the CO dissociation curves. This may or may not be a factor affecting the distribution of CO between the gas phase and the reduced blood after the 30 minute period of shaking required for the present technique of analysis. However, under the conditions outlined above for the absorption of CO by reduced blood, *when ox blood is used*,  $93.7 \pm 2.4$  per cent of known amounts of CO (in concentrations of 0.05 to 0.30 per cent) in air have been recovered from the reduced blood used for the analysis. The corresponding correction factor 1.067 has been found constant to within  $\pm 2.4$  per cent, as is shown in Tables III and IV, and is accordingly used in calculating the factors of Table I.

Arnold *et al.* (1) obtained complete recovery of CO in air exposed to reduced blood. However, they did not control the CO possibly originally present in the blood they used. McIntosh (4), using a Harington (14) modification of the Van Slyke apparatus, controlled the CO content of the human blood used. He assumed that his analytical procedure sufficed "for nearly complete absorption of the carbon monoxide." Nicloux (5) on the other hand, although pointing out the existence of CO in normal blood (3), apparently made no correction for it. Without this control, his eudometric analyses of three gas mixtures ranging from 0.24 to 0.45 per cent of CO in air indicate an absorption of about 93.3 per cent of the CO by beef blood.

## EXPERIMENTAL

In establishing the order of accuracy and the constancy of the results obtained by the technique of analysis outlined above, two sets of experiments were performed.

In the first set, the results of which are given in Table III, CO gas was diluted to a measured extent with CO-free air. The purity of the CO gas was controlled by analyses for CO<sub>2</sub> and O<sub>2</sub> (7, 11) and by ascertaining that the gas could be completely absorbed by Winkler's cuprous chloride solution. Several lots of pure CO were made from formic and sulfuric acid. In none of these preparations was the gas found to be less than 98 per cent pure.

The CO was run into the chamber of the manometric apparatus over mercury, and the pressure (150 to 200 mm.) was measured at 2.0 cc. volume according to the technique (7) used for the measurement of gas samples. For these measurements the gas volume held above the 2 cc. mark was determined by calibrating the chamber with mercury (7), since the volume above a mercury meniscus is greater than above a water meniscus at the same mark. The volume of the CO at 0°, 760 mm., was calculated by multiplying the pressure of the sample at the 2.0 cc. mark by the factor

$\frac{a}{760(1 + 0.00384t)}$  where  $a$  is the volume of gas held by the chamber over a mercury meniscus at the 2 cc. mark.

After the measurement, the gas was completely run over into a large calibrated gas sampling tube of a size to dilute the CO to the desired volume. With negative pressure in the sampling tube, air was admitted until the mixture was just at atmospheric pressure. The sampling tube was then placed in a water bath slightly below room temperature, and again the contents were equilibrated with the atmosphere. The air used was also analyzed for CO by control analyses. At no time was there demonstrable a significant amount of CO in the air used for dilution.

In the second set of experiments, the results of which are recorded in Table IV, the CO mixed with air was measured in a different manner. Fresh ox blood was treated to give it a CO content of 8 to 10 volumes per cent, and the exact CO content was found by analysis by the Sendroy-Liu method. A sample of 1

cc. of the blood was then treated with acidified ferricyanide in the manometric chamber and the liberated CO was extracted. The CO gas thus obtained was diluted by the admission into the chamber, of 45 to 47 cc. of room air. The CO + air mixture

TABLE III  
*Results of Analyses of Air to Which Measured Amounts of CO Were Added Directly*

Sample No.	CO per 100 cc. air		Ratio (a) (b)	Deviation from average ratio 0.937
	Regained from blood (a)	Present (b)		
	cc.	cc.		
1	0.087	0.094	0.925	-0.012
	0.087		0.925	-0.012
	0.086		0.915	-0.022
	0.091		0.968	+0.031
	0.085		0.904	-0.033
2	0.047	0.049	0.959	+0.022
	0.045		0.919	-0.018
	0.047		0.959	+0.022
3	0.181	0.200	0.906	-0.031
	0.181		0.906	-0.031
	0.180		0.900	-0.037
	0.182		0.911	-0.025
4	0.184	0.197	0.920	-0.017
	0.179		0.898	-0.039
5	0.180	0.200	0.900	-0.037
6	0.200	0.209	0.957	+0.020
	0.192		0.919	-0.018
	0.196		0.938	+0.001
	0.185		0.886	-0.051
	0.190		0.910	-0.027

was transferred through the capillary side arm into the gas sample container. After the chamber had been thoroughly cleaned the gas mixture was analyzed according to the procedure given above.

The results of Tables III and IV show that the values for the

ratio  $\frac{a}{b} \left( = \frac{\text{CO found}}{\text{CO present}} \right)$  by the first set of analyses of six different gas mixtures average 3 per cent lower than those found for the second set of analyses of eight gas mixtures. Since there is no reason to doubt the validity of either set of results, equal weight has been given to both, and an average value of 0.937 for the ratio

TABLE IV

*Results of Analyses of Air to Which Were Added Portions of CO Released from Analyzed Blood*

Sample No.	Approximate concentration of CO in gas mixture	CO in air sample		Ratio (a) (b)	Deviation from average ratio 0.937
		Regained (a)	Present (b)		
	<i>per cent</i>	<i>cc.</i>	<i>cc.</i>		
1	0.27	0.0856	0.0900	0.952	+0.015
	0.27	0.0847		0.942	+0.005
2	0.28	0.0883	0.0994	0.935	-0.002
3	0.27	0.0834	0.0853	0.978	+0.041
4	0.26	0.0779	0.0807	0.965	+0.028
5	0.30	0.0941	0.1020	0.922	-0.015
	0.31	0.0971	0.1020	0.951	+0.014
6	0.29	0.0909	0.0975	0.932	-0.005
7	0.27	0.0826	0.0847	0.976	+0.039
8	0.27	0.0853	0.0870	0.981	+0.044

$\frac{a}{b}$  has been derived. The reciprocal of this is the factor 1.067 used in calculating the factors of Table I, to give the actual amount of CO present in analyzed gas mixtures.

#### SUMMARY

A method is described, whereby air containing carbon monoxide in concentrations from 0.05 to 0.3 per cent may conveniently be analyzed in the Van Slyke-Neill apparatus. The CO is

first combined, in the chamber of the apparatus, with the hemoglobin of completely reduced blood, and the CO content of the blood is then determined by the method of Sendroy and Liu.

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## ATYPICAL (SLOW) LACTOSE FERMENTING *B. COLI*

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During studies on infectious diarrhea (Jones and Little (1931)) in cows, motile Gram-negative rods which failed to attack lactose were frequently encountered among the colonies developing on the surfaces of lactose agar plate cultures streaked with fecal suspension. Their numbers varied greatly; at times they made up 90 per cent of the organisms, at others they were present in relatively small numbers and in many cases no such colonies were found. In one instance they were found throughout the small intestine of a cow slaughtered during an attack of diarrhea. At first it seemed to us that we had to deal with organisms of the paratyphoid group but sharp differences in agglutination affinities between these organisms and both types of animal paratyphoid indicated little immunological relationship. In addition, cultural differences were readily manifested.

Many have reported the presence of atypical non-lactose fermenting *B. coli* in the intestinal and urinary tract of man and in a few instances they have been noted in the feces of horses and cattle. Gilbert and Lion (1893), appear to have been the first to describe Paracolon bacilli. Morgan and Ledingham (1909), encountered such types in human feces and divided them into three groups. On the basis of cultural characters they reported them as numerous in cases of diarrhea but regarded them as of little pathogenic significance. On the other hand, György (1920) suggested that they played a considerable part in diarrhea in both man and calves. Stewart (1926), who studied the Mendelian variation in organisms of this group, recognized that two distinct types existed: (1) paracolon bacilli, which fail to attack lactose



after long periods, and (2) mutable colon bacilli which possess a routine Mendelian variation with respect to the fermentation of such substances as lactose, sucrose and dulcitol. In this respect Stewart broadened the original definition of *B. coli-mutable* which Neisser (1906) and Massini (1907) applied to the organisms whose action on lactose only was variable.

#### THE SOURCE OF ATYPICAL COLON BACILLI

In our earlier studies on infectious diarrhea of cows we encountered in the feces actively motile organisms which, on the surface of lactose agar plates, failed to attack the carbohydrate. In certain outbreaks all specimens of feces contained them while in others their appearance was irregular and in later epidemics during 1930 and 1931 we failed to find them. In addition to our own isolations, Dr. W. A. Hagan (1925), Professor of Bacteriology at the New York State Veterinary College, Cornell University, supplied us with several strains which he had obtained from intestinal material, pond water, etc., during his study of Braken poisoning of cattle. Furthermore, we examined the feces from 50 normal cows drawn from 5 herds in this vicinity and cultivated atypical colon bacilli once. Another culture was isolated from the urinary tract of a healthy calf. From this it would appear that such organisms are not present in large numbers in the intestinal tract of normal cows although they may be the predominating types during certain intestinal disorders; but, as we have shown, there is little evidence to incriminate them as the etiologic agent in such maladies.

#### THE CULTURAL CHARACTERS OF THE GROUP

When the surfaces of agar plates, containing brom-cresol-purple, in addition to 1 per cent lactose, are streaked with feces or culture, smooth round translucent colonies of actively motile bacilli developed. The surrounding medium retains its purple color even after incubation or storage in the room for two weeks or more. In our hands, buds or protrusions which ferment lactose have not occurred, although strains may, as will be shown later, under certain conditions, develop both rapid lactose and

slow lactose fermenters. When digest broth containing carbohydrate and indicator (brom-cresol-purple) is inoculated with culture, many of the carbohydrates (table 1) are promptly fermented with acid and gas production. Lactose broth, however, remains alkaline for varying periods. When the cultures were freshly isolated, there was no appreciable change in reaction during the first three or four days of incubation and, in some cases, during the first two or three weeks. One strain isolated from the urine failed to attack lactose, as judged by acid production and

TABLE 1  
*The biochemical characters of atypical colon bacilli*

NUMBER OF STRAINS	GLUCOSE	LACTOSE	SUCROSE	MALTOSE	MANNITOL	XYLOSE	RAFFINOSE	SALICIN	DULCITOL	VOGES-PROSKAUER	INDOL	METHYL RED	LIQUEFACTION OF GELATIN	PRODUCTION OF H <sub>2</sub> S
7	A.G.	Slow fermentation	A.G.	A.G.	A.G.	A.G.	0	A.G.	A.G.	-	+	+	-	-
1	A.G.	Slow fermentation	A	A.G.	A.G.	A.G.	0	A.G.	A.G.	-	+	+	-	-
1	A.G.	Slow fermentation	A.G.	A.G.	A.G.	A.G.	0	A.G.	0	-	+	+	-	-
2	A.G.	Slow fermentation	A.G.	A.G.	A.G.	A.G.	0	0	A.G.	-	+	+	-	-
12	A.G.	Slow fermentation	0	A.G.	A.G.	A.G.	0	A.G.	A.G.	-	+	+	-	-
15	A.G.	Slow fermentation	0	A.G.	A.G.	A.G.	0	A.G.	0	-	+	+	-	-

gas formation, during thirty days. However, after repeated transfer it now begins to ferment this substance after four or five days. As is brought out in table 1, all strains readily attacked glucose, mannitol, maltose, and xylose, with the production of acid and gas, and failed to ferment raffinose, but fermented lactose slowly. In addition all gave a negative Voges-Proskauer reaction, produced indol, gave a positive methyl-red reaction, and failed to liquify gelatin or produce H<sub>2</sub>S. In the fermentation of such substances as sucrose, dulcitol, and salicin, differences were noted as indicated in table 1.

From the data in table 1, it appears that of the 38 strains the majority (27) fell into one group when judged by inability to attack sucrose; this large group could again be subdivided on the basis of dulcitol fermentation since 12 fermented this carbohydrate and 15 failed to do so. Eight of the 11 strains which attacked sucrose fermented both dulcitol and salicin. Of the other 3 sucrose fermenters, 2 fermented dulcitol but not salicin and the other the reverse.

Our cultures, then, could be divided into two groups on the basis of sucrose fermentation. The larger, which failed to attack sucrose, could be subdivided into two subgroups based on the property of attacking dulcitol. The smaller group, all strains of which fermented sucrose, was more complex in that it contained individuals which differed in their behavior in broth containing dulcitol and salicin.

The apparent failure of the organisms to utilize lactose when grown on the surface of lactose agar plates aroused our interest. Several facts were observed in this connection. The cultures grew well on the lactose plates. When true *B. coli* was seeded on the same plate with atypical strains, it readily attacked the sugar. The results then could not be attributed to poor growth or alteration of the carbohydrate. Further, the odor of the culture was different. *B. coli* was characterized by a sour odor, while the atypical type produced a strongly disagreeable ammoniacal one. When Durham tubes containing 1 per cent lactose are seeded with culture even after a year or more of cultivation, growth begins promptly but the indicator remains unchanged for two or more days. Acidity is first noted at the bottom of the tube and the reaction gradually changes, accompanied by a moderate gas production. Ten days or more may be required to change the color of the indicator completely. When lactose agar plates are seeded and incubated anaerobically the result is the same as when the plates are incubated in the air; the reaction of the medium is unchanged. Furthermore when certain strains of true *B. coli* of bovine origin are grown on the surface of lactose agar plates the medium is promptly acidulated. If, however, growth is artificially checked by a sharp reduction of temperature

the medium becomes alkaline after a few hours, but the onset of optimum growth conditions will again inaugurate acid production. There exist then among the true colon bacilli races which under optimum conditions readily attack lactose with the formation of acid, but during retardation of growth produce sufficient alkali to shift the pH toward the alkaline side.

That perhaps there existed in our cultures of atypical colon bacilli some such balance between carbohydrate attack and alkali production seemed plausible and the following observation was designed to test this possibility.

Sugar free digest broth, pH 7.2, was distributed in amounts of 50 cc. into 100 cc. flasks. Sufficient 20 per cent lactose was added to make a concentration of 2 per cent. The flasks were inoculated in pairs with 0.2 cc. of an eighteen-hour broth culture of the organism. Half of the flasks were incubated at 38°C., and half at 22°C. The series included in table 2 contained 5 cultures of the slow lactose fermenting organisms, 1 *B. coli* which, when once it had acidulated the medium of a lactose agar plate culture, never became alkaline, and another which readily produced acid under optimum growth conditions but during retardation produced alkali. In addition the sterile medium was held for control purposes. After two-and four-day intervals the amount of lactose was quantitatively determined by means of Benedict's solution. The hydrogen ion concentration was determined and the effect of the addition of formol on pH was recorded. The results are recorded in table 2.

These observations were repeated on 3 occasions with similar results. From the data in table 2, several points are readily recognized. First, that the atypical colon bacilli utilize lactose from the start. It is true that at 38°C. the utilization is not so marked as that of typical colon types. On the other hand the amount of lactose utilized at 22°C. is comparable with that consumed by *B. coli* under the same condition. Another significant point is the effect of lactose consumption on pH. This is best illustrated in the titrations after four days of growth at 22°C. In the case of the atypical cultures, the utilization of lactose is comparable to that of colon bacilli. Nevertheless, the acidity is

relatively low, varying between pH 6.8 and 7.4, whereas under the same conditions *B. coli*, while consuming no more sugar, has shifted the pH to 5.2 in one instance and 4.4 in the other. The addition of the same quantity of neutral formol to all cultures likewise reveals sharp differences. When added to the *B. coli* cultures it either had no effect (i.e., failed to reduce the final pH of 4.4) or depressed it to 4.7. The same treatment of the atypical culture revealed about the same buffer content as that possessed by the original medium, as indicated by a shift to pH 5.9. In

TABLE 2

*The utilization of lactose and acid production by various cultures*

CULTURE	2 DAYS						4 DAYS					
	38°C.			22°C.			38°C.			22°C.		
	Lac- tose uti- lized	pH	pH after addi- tion of formol	Lac- tose uti- lized	pH	pH after addi- tion of formol	Lac- tose uti- lized	pH	pH after addi- tion of formol	Lac- tose uti- lized	pH	pH after addi- tion of formol
	mgm.			mgm.			mgm.			mgm.		
Atypical 3497-K....	6.6	6.0	5.0	7.0	7.0	6.8	11.5	6.4	5.2	8.0	7.4	5.9
Atypical A-74.....	9.0	7.8	6.0	8.4	7.4	5.8	9.5	5.9	5.4	9.3	6.8	6.0
Atypical 182.....	7.7	7.2	6.0	9.7	7.0	5.8	9.0	7.8	5.9	10.0	7.4	5.8
Atypical 894.....	7.4	7.0	5.6	7.0	7.0	5.8	9.0	6.8	5.4	10.9	6.8	5.9
Atypical brom 5-7..	7.6	6.9	5.9	6.8	7.4	5.9	8.2	5.6	4.4	11.0	7.0	7.0
<i>B. coli</i> S H 2.....	10.5	4.4	4.4	10.0	5.0	4.4	12.8	4.4	4.4	11.0	4.4	4.4
<i>B. coli</i> 1736*.....	9.8	5.0	4.4	5.7	5.6	5.0	12.5	4.4	4.4	8.0	5.2	4.7
Uninoculated media.		7.4	5.9								7.4	5.9

\* Acid during rapid growth on lactose agar plate but alkali when retarded.

the instance of culture brom 5-7, it required the addition of twice the amount of formol to produce this result.

There exists, then, experimental evidence that the atypical colon bacilli possess the property of utilizing lactose but at the same time produce alkali, for a time at least, in sufficient quantities to mask the acidity of fermentation.

#### THE IMMUNOLOGICAL PROPERTIES OF ATYPICAL COLON BACILLI

Rabbits were immunized with several strains of typical colon bacilli and with the atypical strains. In general, little relation-

ship existed between individual strains even when drawn from the same source, as illustrated by the results recorded in table 3. The serum was prepared by immunizing a rabbit with strain 182-II and titred against other cultures identical in cultural characters and from the same plate culture.

All cultures were tested against various sera of both the typical and atypical types drawn from the same sources but no definite grouping could be established.

Stewart (1926) commented on the fact that the cultures with which he worked possessed the property of acquiring certain fermentative characteristics. This was established by observ-

TABLE 3

*Illustrating the heterogenous agglutination affinities of atypical B. coli cultures obtained from the same material*

CULTURE	SERUM DILUTIONS									C
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	
Homologous	C	C	C	++++	+++	+++	++	++	+	-
182 II										
182 II-0	++	+	±	-	-	-	-	-	-	-
182 II-I	C	C	+++	++	+	-	-	-	-	-
182 II-02	+	++	+	+	+	+	±	±	±	-
182 III	C	C	++	+	+	+	+	±	±	-
182 IV	++	+	-	-	-	-	-	-	-	-
182 II 40	+	++	-	-	-	-	-	-	-	-

ing colonies which produced buds along their borders. The organisms of the buds frequently acquired the property of fermenting the carbohydrate, an attribute not possessed by the bacteria of the original culture. This has not been true with our cultures. When buds have been formed they behave toward the environment (lactose) as did the parent colony. Many cultures were rapidly passed through lactose broth, and of these a number developed two types of colonies. One type produced acid when growing on the surface of lactose agar plates and the other behaved in all respects like the atypical culture. Certain of our strains then possessed two elements both capable of fermenting lactose but one presumably producing sufficient alkali to mask acidity and the other apparently lacking this property.

On the other hand, once a prompt lactose fermenting type had been obtained from an atypical strain this character became fixed and rapid transfers through media containing no sugar, or rich in blood serum, never produced a reversion to the atypical form.

There were then two components in some of our strains. One, which when cultivated in a proper environment, was a true colon bacillus; the other in which the dominant factor was the atypical type.

It seemed of interest to show whether or not such a change had altered the antigenic nature of any of the forms. For this purpose 2 series of cultures were chosen, but only 1 series of experiments is reported in detail. The parent types consisted of subcultures carried on in the usual manner, the others were selected after rapid passage through broth containing lactose; those that promptly attacked lactose after several passages are referred to as Y, and those that still failed to do so under these conditions, as B. Individual rabbits were immunized with parent A, Y and B strains and the various sera tested with all the strains. Each serum was tested with both homologous and heterologous cultures. The initial titers of the various sera produced from strain 1306 are given in table 4.

The parent strain and the others possess certain antigenic properties in common as judged by agglutination. The slow lactose fermenting B type agglutinates much better in all sera than any of the others. The character of the clumps differed sharply. B always produced the fluffy type of flocculation and the others the granular type. For a clearer understanding of these differences further experiments were necessary.

The first step consisted in the usual absorption tests. Sufficient of the heterologous strain was added to reduce greatly the agglutinin for the homologous strain. The results of this procedure are shown in table 5.

On the whole the results were unexpected. The parent strain A and the rapid lactose fermenting type Y behave the same, each absorbing agglutinin for the other. The results with the B type, which utilized lactose slowly, appear to suggest that a new antigenic entity appeared during the course of selection, but certain





data belie this. It is recorded in table 4 that the antiserum produced by both the A (parent) and Y strain agglutinated the B culture better than the homologous strains. Therefore, both A and Y must have contained sufficient antigens similar to B to produce B antibody. That both A and Y strains possessed relatively little of such antigen can be argued from the fact that even when sufficient A and Y culture was used to reduce greatly the agglutinin for these strains the agglutinin for B remained relatively unaffected. Culture B seemed to possess the property of absorbing agglutinin for both A and Y.

TABLE 6

*The effect of massive absorption of serum 1306 upon the agglutinin*

SÉRUM	STRAIN	SÉRUM DILUTIONS						
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
Unabsorbed serum.....	A	C	C	++++	++	+	+	+
	B	C	C	C	C	C	C	C
	Y	C	C	++++	++	+	+	+
Absorbed with massive quantities of strain A.....	A	++	+	±	±	+	+	+
	B	++	+	+	+	+	+	+
	Y	++	+	+	+	+	+	+
Absorbed with massive quantities of strain Y.....	A	++	++	++	+	++	++	+
	B	++	++	++	+	++	++	+
	Y	++	++	++	++	++	++	+

If, then, strains A and Y possessed the dominant antigen for B in small quantities only it should be possible to remove agglutinin from these antisera provided large enough quantities of culture were used for absorption. This proved to be the case, as brought out in table 6.

Antiserum A was diluted 1:10 with NaCl and was repeatedly absorbed with massive quantities of either A or Y strains. As an instance, the A serum was absorbed on 6 occasions with all the bacteria obtained from the surface of 9 Blake bottles of culture Y. Another portion of A serum was absorbed with large quantities

of homologous culture. The effects of such absorption on the agglutinins for B are shown in table 6.

It is evident then that the parent A strain and the active lactose fermenting Y type actually possess B antigen in sufficient quantity to produce antibody. The quantity is so small as hardly to affect the agglutination titer when the serum is absorbed by moderate quantities of A and Y cultures, but when large numbers of these organisms are employed for absorption the B agglutinin is removed. The apparent antigenic difference then is quantitative and it appears that A and Y possess similar antigens to B but in B the antigens which produce the fluffy agglutination are present in greater quantities.

In another less complete experiment both the Y and B strains developed similar antigenic qualities and agglutinated better in all sera than the parent strain.

#### DISCUSSION

We have described certain characteristics of a type of colon bacilli encountered in many cases of infectious diarrhea in cows. In other papers we have shown that these organisms, although frequently predominating in the feces and readily obtained from various portions of the intestinal tract of cows suffering from diarrhea, cannot be considered as the cause of the disease. Our experiments indicated that when fed to calves the organisms, either as pure cultures or fecal suspensions failed to establish themselves in the intestinal tract. In three instances cases were followed from early in the course of the disease until recovery. At the height of the attack the organisms in question were present in the feces in large numbers, during convalescence their numbers diminished, and they could no longer be found when health was restored.

From their appearance on lactose agar plates these organisms may readily be mistaken for paratyphoid bacilli especially when freshly isolated and cultivated in glucose, lactose, and sucrose. The relative tardiness with which they ferment lactose is at first deceptive although they are readily differentiated from the paratyphoids by agglutination and more complete cultural study.

We prefer to designate the organisms with which we worked as atypical colon bacilli in contra-distinction to the mutable and para types of Stewart. Our organisms invariably attacked lactose in contrast to the para type of Stewart and differed from the mutable type since it was not possible to show that they acquired new fermentative properties. On the basis of our data it seems preferable not to regard them as true mutants since we were unable to show that new characters had been developed or original properties had been lost. We have called attention to certain races of colon bacilli which readily attack lactose under optimum growth conditions and as readily produce alkali when such conditions are abruptly changed. There exists naturally a race of *B. coli* which utilizes carbohydrate under certain conditions and presumably attacks nitrogenous matter under different conditions. Our strains possess both properties. Although they utilize the carbohydrate (lactose) a little more slowly than do true *B. coli*, nevertheless during this phase they form sufficient alkali, probably ammonia, to stabilize the reaction of the media, and hence the indicator retains its original color. By rapidly passing certain strains through lactose it has been possible to obtain 2 types, one of which attacks lactose with ordinary rapidity and is essentially a true colon type and the other, which is the atypical organism. The former has not reverted to the atypical form in our hands. If, then, the atypical organism is one which differs from the typical quantitatively in its ability to produce more alkali and perhaps to utilize a little less lactose, its appearance in the intestine in large numbers under abnormal conditions might be explained on the grounds of a more favorable environment during illness which facilitates the growth of this type. The lack of proper conditions might explain the apparent rarity of such organisms during health and their disappearance from the feces with a return to normal.

Certain of the immunological findings bear out the contention that members of the group differ only in degree, rather than constitutionally. The experiments reported for culture 1306 strongly support this view. Here, the parent strain and one of the derivatives which readily attacked lactose possessed identical

agglutination affinities. The slowly fermenting lactose culture apparently possessed a different antigenic complex. Further experiments, however, indicated that apparent differences could be explained on quantitative grounds, so that in reality there were no appreciable antigenic differences.

The organisms are of interest to those concerned in the matters of public health since they may readily gain access to milk from the feces and at first be mistaken for paratyphoid bacilli.

#### SUMMARY

This paper deals with the cultural characteristics of atypical colon bacilli frequently encountered in the feces of cows suffering from intestinal disorders. In lactose agar plate cultures the organisms fail to change the reaction of the media and for this reason they may be mistaken for paratyphoid bacilli. It has been established that in broth containing lactose there is a phase in which the carbohydrate is utilized without changing the reaction of the medium, followed by a phase in which acid is slowly produced. Such organisms are not regarded as true mutants of *B. coli* since we were able to show that no new qualitative differences had developed but that apparent cultural and antigenic differences could be explained on quantitative grounds.

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## GENETIC CRITERIA OF BREEDING WORTH IN DAIRY SIRES

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Animal breeding studies on dairy cattle have one common objective—to establish facts which will lead to the understanding of the influence of heredity and environment on production. The herd sire is the center of this problem since his germ plasm is so extensively multiplied within the herd. It is important to know what this bull's daughters will produce, not after they have a milk record as at present, but before the mating is made from which they are to be born. It is proper in a great congress gathered to discuss the vital problems of this great industry to inventory the status of this paramount problem.

Several methods of estimating the sire's worth as a transmitter of milk production have been suggested. The first of these methods in point of historical time is no doubt the average of the sire's daughters' records, at first probably not corrected for age but now with a better understanding of its importance, corrected for age. This test of the sire's worth has been most extensively put to practical use under the title of the sire's progeny performance (Gowen).

Sire's progeny performance = Average age-corrected daughters' records,  $\bar{O}$ . (1)

This measure, while by no means considered ideal, furnishes valuable, if incomplete, information. It is the measure for which most data are available. It has the advantage of being closely related to the economics of production for if a bull's daughters do not produce well they will be uneconomic and the bull purchased a worse calamity than the appearance of some dread epidemic in the herd. If, on the other hand, they do have a high average  $\bar{O}$ , the herd prospers and the bull will be of very real value to the herd. The value  $\bar{O}$  taken in conjunction with the variation of the daughters' productions is a direct measure of the quality of the inheritance which the sire has to transmit for milk produc-

tion. It needs, however, added information to complete or extend its worth as a breeding or inheritance index.

The most obvious information of possible importance to the sire's progeny performance,  $\bar{O}$ , is the record of the sire's mates. Several different formulae have been derived to measure the sire's future breeding worth utilizing these two facts, the average age-corrected daughters' records  $\bar{O}$ , and the records of the sire's mates  $\bar{D}$ . In point of historical time, the first of these measures was the difference between the sire's daughters' age-corrected records and the age-corrected records of the sire's mates (Eckles).

$$\text{Sire's daughter-dam index} = \text{Average daughters' age-corrected production} - \text{average dam's age-corrected production, } \bar{O} - \bar{D}. \quad (2)$$

This measure is a purely objective measure. It is an important measure from the viewpoint of the owner of the bull since it would appear beyond possibility of doubt that if a bull's daughters are on the average poorer milkers, or poorer in the quality of their milk, than the dams from which they came, then the bull is exercising a harmful effect upon the herd. On the other hand, if the bull's daughters are on the average measurably better than the dams from which they came, in productive qualities, then that bull is exercising a beneficial effect on the herd of which he is the head, the production of the herd is rising and the owner is on a better economic plane. This index is, however, of use in conjunction with the sire's progeny performance,  $\bar{O}$ , rather than of use separately (Gowen). The formulae  $\bar{O}$  and  $\bar{O} - \bar{D}$  were suggested before any extensive knowledge of inheritance of milk production was available.

With the results of definite mating of high and low producing strains of cattle and studies within different breeds becoming available, other formulae for the sire's breeding worth were evolved utilizing the facts of these studies in their derivation.

Studies on the Jersey, Guernsey and Holstein-Friesian breeds of cattle lead to the conclusion that the inheritance of milk production is no different than that of other characters. It is of the Mendelian multiple factor type. The degree of determination of the milk production of cow by heredity varies with the breed but is of the order of 70 per cent or slightly more. The degree of determination for butter-

fat percentage is higher, at least 80 per cent and perhaps more. The effect of the environment common to two pairs of tested animals has an effect of the order of 5 per cent on the average although in some cases 10 per cent and with some evidence that it may go higher in special cases. The correlation coefficients for the daughters and their dam's productions range from .30 to .50 for the 3 breeds; the Jersey the lowest, the Holstein-Friesian the highest. For the butter-fat percentages the correlations are 0.41, 0.42 and 0.42 for these breeds. The half-sister correlations range from 0.13 to 0.38 for milk yield and 0.17 to 0.37 for butter-fat percentage. The average for the sire's and dam's daughters milk yields is the same 0.24. The average for the sire's daughters' butter-fat percentages is 0.26 and for the dam's is 0.20. The average correlation of sire and daughter indirectly determined and known to be slightly too high is 0.55 for milk yield and 0.53 for butter-fat percentage. For dam and daughter the average correlations directly determined are 0.39 for milk yield and 0.41 for butter-fat percentage. These correlations are shown tabulated below:

TABLE I

*Correlation Coefficients for Milk Production and Butter-Fat Percentage within the Advanced Registries of the Breeds*

Correlated pairs	Breed					
	Holstein-Friesian		Guernsey		Jersey	
	Milk	Butter-fat percentage	Milk	Butter-fat percentage	Milk	Butter-fat percentage
Sire and daughter.....	0.52	0.53	0.50	0.54	0.63	0.51
Dam and daughter.....	.50	.41	.36	.42	.30	.42
Full sisters.....	.55	.46	.41	.44	.39	.41
Half sisters (sire).....	.36	.37	.13	.17	.23	.25
Half sisters (dam).....	.38	.22	.15	.19	.20	.20

These correlations and the facts derived from them furnish the basis for the following assumptions. First, that inheritance of milk yield and butter-fat percentage is Mendelian in character, the factors sorting almost at random so far as milk yield and butter-fat percentage are concerned. Second, that the correlations of parent and offspring may



be considered 0.5 and between siblings 0.25. Heredity is the most important single variable determining production.

On the basis of these assumptions, Wright has made the following important contribution to the indices for evaluating the sire's breeding worth:

$$S = A + \frac{n}{n+2} (2 \bar{O} - \bar{D} - A). \quad (3)$$

Where  $S$  = sire's transmitting ability,  $A$  = breed's average production,  $n$  = number of daughter-dam pairs,  $\bar{O}$  = average daughter's age-corrected production and  $\bar{D}$  = average production of the sire's mates.

Another index which was suggested independently by Hansson and by Woodward and later by Yapp comes directly from this formula of Wright's by considering  $n$ , the number of daughters infinite. The sire's index under these conditions equals:

$$S = 2 \bar{O} - \bar{D}. \quad (4)$$

Other modifications of this formula have been suggested, dependent on the particular assumptions with regard to the effects of heredity, environment, etc.

A fifth index has been obtained utilizing another aspect of the data on the inheritance of milk production. In crosses of different breeds of cattle which have characteristically distinct milk productions or butter-fat percentages the results show that a given level of milk yield or butter-fat percentage is not completely dominant, since the offspring are intermediate between the parental levels in their productions<sup>1</sup>). Goodale perceived the significance of these facts and from them formulated the fifth important sire's index. On an age-corrected basis, the sire's breeding index for milk yield is the sire's mates' average production plus 1.4 times the difference between the dams' and daughters' average when the latter is greater. When the daughters' production is less than the dams' then 3.3 times the daughters-dam difference is subtracted from the dam's average to get the

<sup>1</sup>) Milk Secretion, Williams & Wilkins Co., Baltimore, Maryland, U. S. A., pp. 349-355.

sire's index. For the butter-fat percentage the multipliers are 2.5 and 1.7 respectively.

These are the major criteria for a sire's breeding ability where the information is limited to the sire's mate's records and the records of his daughters. For convenience in tabling they may be represented as  $\bar{O}$  for equation 1,  $\bar{O}-\bar{D}$  for equation 2,  $S$  for equation 3,  $2 \bar{O}-\bar{D}$  for equation 4 and  $G$  for Goodale's index.

There are two major problems which are involved in evaluating the breeding worth of any dairy sire. The owner, studying his own bulls to determine their relative merits for continued use within his herd, presents one aspect of the problem. The owner intending to go outside of his herd for the purchase of a bull for use in his herd presents the other phase of this problem. In the first case the bull has all the environmental elements as herd management, relative production of cows etc. continued as in the past. In the second case all of these environmental factors will change. A test of the relative merits of the various formulae under these conditions may be had by determining the various sire's indices on past known data and then determining what relation exists between these indices and the production of daughters which came after the time of the daughters on which the indices for the sires' were made. The criterion by which the efficacy of the various indices will be measured is its accuracy in indicating the milk yield or butter-fat percentage of the offspring, unknown at the time the estimate of the sire's breeding capacity is made.

For this purpose we shall use the records of the Jersey Registry of Merit. The five sire's breeding indices,  $\bar{O}$ ,  $\bar{O}-\bar{D}$ ,  $S$ ,  $2 \bar{O}-\bar{D}$  and  $G$ , were calculated on the basis of the Jersey Registry of Merit data earlier than 1923. These were used to predict the production of their offspring which made their first record in a period so that it appeared in the Registry of Merit books later than 1923. In large part these daughters were born, raised and tested in the same herd as that in which the sire was previously tested. This procedure is entirely like predicting the production of a sire's unborn daughter and then comparing this predicted production with that which she actually gave when she came into milk. The best measure of such a comparison is the correlation coefficient as this measure takes into account all possible combinations properly weighted, and gives the result as a

single numerical figure in easily understood terms. The data are given in Table II.

TABLE II

*Correlation Coefficients Between Sires' Breeding Indices and Their Daughters' Actual Productions, 545 Daughters*

Index	Correlation coefficients with later daughters	
	Milk yields	Butter-fat percentage
$\bar{O}$	$0.433 \pm 0.023$	$0.482 \pm 0.022$
$\bar{O}-\bar{D}$	$.176 \pm .028$	$.332 \pm .026$
S	$.345 \pm .026$	$.432 \pm .023$
$2\bar{O}-\bar{D}$	$.340 \pm .026$	$.456 \pm .023$
G	$.323 \pm .026$	$.448 \pm .023$

The second type of data was obtained by selecting out those sires which had been used in more than one herd and determining the correlations between the sire's indices, derived from the herd with most daughters and the average production of his daughters for each subsequent herd in which he was used. These correlation coefficients are given in Table III.

TABLE III

*Correlation Coefficients between Sire's Breeding Indices and Their Subsequent Daughters' Average Productions, Different Herds*

Index	Milk yield	Butter-fat percentage
$\bar{O}$	$0.213 \pm 0.028$	$0.337 \pm 0.027$
$\bar{O}-\bar{D}$	$.063 \pm .030$	$.128 \pm .029$
S	$.215 \pm .028$	$.277 \pm .027$
$2\bar{O}-\bar{D}$	$.152 \pm .028$	$.273 \pm .027$
G	$.153 \pm .028$	$.246 \pm .027$

The comparison of these two tables brings out clearly the influence of environmental variations on the daughters' production. Throughout the correlations are lower, probably significantly lower, in Table III than in Table II. They are lower for milk yield than they are for butter-fat percentage, pointing to the fact that more attention has been paid to the breeding for milk production than there was to

the breeding for butter-fat percentage, although both are equally controlled by inheritance and amenable to its selection.

The conclusions to be drawn from these two aspects of the sire's breeding indices are the same in several respects. In either case the results show that the simplest index, the sire's progeny performance,  $\bar{O}$  indicates the unknown daughter's productivity most clearly. The index  $S$  appears to be of next importance. The  $\bar{O}$ - $\bar{D}$  index is the poorest of all. All of the index figures indicate something of the unknown daughter's probable production, however, this appears to be due to the fact that all of the index values depend largely on  $\bar{O}$  in their determination. The correlations describing these relations are shown in Table IV.

TABLE IV

*Correlation Coefficients between the Production Records of  $\bar{O}$ ,  $\bar{D}$  and the Unknown Daughter's Yield,  $Da$*

Constant	Milk yield	Butter-fat percentage	Unlike herds	
			Milk yield	Butter-fat percentage
Mean				
$\bar{O}$	10645	5.43	10362	5.35
$\bar{D}$	10110	5.36	10108	5.33
$Da$	11529	5.36	10263	5.38
Standard deviation				
$\bar{O}$	1544	0.353	1902	0.394
$\bar{D}$	1594	.289	1755	.373
$Da$	2775	.549	2378	.499
Correlation coefficient				
$\bar{O} \bar{D}$	$0.447 \pm 0.022$	$0.445 \pm 0.022$	$0.404 \pm 0.024$	$0.486 \pm 0.023$
$\bar{O} Da$	$.433 \pm .022$	$.482 \pm .021$	$.213 \pm .028$	$.337 \pm .027$
$\bar{D} Da$	$.249 \pm .026$	$.215 \pm .026$	$.163 \pm .028$	$.182 \pm .029$
$\bar{O} Da, \bar{D}$	$.372 \pm .025$	$.441 \pm .022$	$.163 \pm .028$	$.326 \pm .027$
$\bar{D} Da, \bar{O}$	$.068 \pm .030$	$.001 \pm .030$	$.086 \pm .029$	$.022 \pm .030$

The partial correlations of  $\bar{O}$  and  $Da$  and  $\bar{D}$  constant and of  $\bar{D}$  and  $Da$  with  $\bar{O}$  constant strengthen the conclusion that the sires' mates play little part in determining the productivity of the sire's daughter,  $Da$ , in the Jersey Registry of Merit data.

The constants for the sire's progeny performance,  $\bar{O}$ , the sire's mate's yields and the daughter's productions lead to the following equations:

Sire used largely in same herd.

Daughter's milk production =  $2624 + .72 \bar{O} + .12 \bar{D}$ .

Daughter's butter-fat percentage =  $1.27 + .75 \bar{O} + .014 \bar{D}$ .

Sire used in different herds.

Daughter's milk production =  $6223 + .22 \bar{O} + .13 \bar{D}$

Daughter's butter-fat percentage =  $3.00 + .41 \bar{O} + .03 \bar{D}$ .

These equations bring out clearly the smallness of the effect of the sire's mates on the milk production or butter-fat percentage of a subsequent unknown daughter in the Jersey Registry of Merit cattle. This conclusion can also be reached from the fact that the correlation coefficient of daughter and dam is rather lower than expected on random mating and multiple Mendelian factor grounds and the size of the correlation between the sire's mates. These correlations are  $0.19 \pm .03$  and  $0.18 \pm .03$  taking the sire's mates as they come (including 980 pairs of repetitions of identical cows in 29,638 pairs) or  $0.17 \pm .03$  for milk yield and  $0.15 \pm .03$  for butter-fat percentage when only unlike pairs are used. The correlations between the sire's mates average productions and those of her own dam are also rather large,  $0.21 \pm .03$  for milk yield and  $0.25 \pm .03$  for butter-fat percentage. These relations lead to a relatively small effect of the sire's mates in indicating what the coming daughters production will be, less than a tenth and decreasing with the number of daughters on which the sire's breeding worth is based.

The data also bring out the differentiation which exists between the different herds with respect to their milk producing capacity. This differentiation is apparently due to both genetic and environmental causes. The comparison of the correlation coefficients cannot be made exactly, however, because the like herd comparison is for the cows later than 1923 and the unlike herd comparison is for cows earlier than 1923.

The last factor which may be considered should on theoretical grounds certainly have some importance as it makes for a more adequate breeding test of the sire and therefore a better prediction of the

performance of his future daughters. This factor is the number of daughters on which the sire's progeny performance is based. Study of this question in the Jersey cattle does not indicate that this factor plays any large part. The correlation coefficients for the sire's progeny performance and the future daughters' records for certain groups of  $n$ , the number of daughters on which the sire's progeny performance was based, are shown in Table V.

TABLE V

*Correlation Coefficients between Sire's Progeny Performance,  $\bar{O}$ , and His Future Daughters' Productions for Different Classes of  $N$ , the Number of Daughters on Which the Sire's Progeny Performance was Based*

Sires used in like herds			Sires used in different herds		
$n$	Milk yield	Butter-fat percentage	$n$	Milk yield	Butter-fat percentage
2	$0.49 \pm 0.07$	$0.31 \pm 0.08$	1	$0.13 \pm 0.06$	$0.31 \pm 0.05$
3	$.43 \pm .07$	$.67 \pm .04$	2	$-.03 \pm .07$	$.43 \pm .07$
			3	$-.07 \pm .12$	$.26 \pm .11$
4	$.50 \pm .08$	$.38 \pm .09$	4 or 5	$.32 \pm .06$	$.29 \pm .06$
5 or 6	$.40 \pm .09$	$.32 \pm .09$	6 and over	$.40 \pm .05$	$.37 \pm .05$
7, 8, or 9	$.33 \pm .06$	$.41 \pm .06$			
10 or more	$.49 \pm .04$	$.33 \pm .05$			

Table V shows that in these Jersey cattle the correlations do not show any pronounced change with the increase of the number of daughters on which the sire's progeny performance was determined where the sire is continued in the same herd. Where the sire changes herds the correlation for milk yield increases with the number of daughters on which the sire's progeny performance was based. In the butter-fat percentage group the correlations may possibly increase somewhat but the evidence is by no means clear that it does.

The case of the sire's transferring from one herd to another rep-

resents the situation which is most frequently found. It is in general this evaluation of the sire's breeding worth which is desired. The data here presented show that practically all dependence is to be placed in the sire's progeny performance,  $\bar{O}$ , for obtaining this evaluation. This in conjunction with the average of the breed gives the best estimate of production. The constants necessary for this equation may be obtained from the correlations, daughter and dam, the sire's mates, and daughter's dams and sire's mates. These differ somewhat for milk yield and for butter-fat percentage but considering the errors involved may be considered as average values between the two, daughter and dam 0.35, sire's mates 0.16 and daughter's dam and sire's mates 0.22. Using the technique of path coefficients of Wright, these equations lead to the awkward expression:

Sire's genetic worth =

$$A + \frac{n}{6.2 + n} [\bar{O} - A] + \frac{(1.3 + .13n) n}{(5.2 + n) (6.2 + n)} [\bar{D} - A]$$

The last term  $\frac{(1.3 + .13n) n}{(5.2 + n) (6.2 + n)} [\bar{D} - A]$  can for most practical

purposes be neglected as its coefficient is never more than a fourth of  $\bar{O}$ . It will be noticed that this term comes out a plus value rather than the negative value of the other formulae for the sire's breeding worth. The correlations which may be derived from this formulae are approximated by those obtained in the last two columns of Table V.

#### SUMMARY

This paper presents a study of different measures of determining a sire's breeding worth. The analysis shows that at the present time the measure best adapted to common use is the sire's progeny performance,  $\bar{O}$ , since it accurately predicts the future daughters production as any measure and since it is the simplest to calculate. This conclusion is supported by the analysis of the relations which exist between the variables involved  $\bar{O}$ ,  $\bar{D}$  and  $n$  and  $Da$ . Sire's genetic

worth = breed average production +  $\frac{n}{6.2 + n}$  [Sire's progeny performance—breed average production].

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## COLEOPTERA AND DIPTERA COLLECTED FROM A NEW JERSEY SHEEP PASTURE

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Domestic animals are known to harbor large numbers of parasitic worms, some species of which, notably among the tapeworms, have unknown life histories with probable intermediate stages in insects. Hall (1929) in a recent paper on "Arthropods as Intermediate Hosts of Helminths" lists thirty-four species of insects which serve as intermediate hosts of Cestoda, fifty-three for Trematoda, one hundred and forty-one for Nematoda, and fifteen for Acanthocephala. Members of the orders Coleoptera and Diptera appear most frequently in such a list, with Siphonaptera, Lepidoptera, Odonata, Mallophaga, Dermaptera, Trichoptera, and Ephemera also represented. Insect collections with special reference to those found in the dung of various domestic animals, thus serve as a method of orienting research on the life histories of certain of the helminths parasitizing such animals.

During the summer of 1928, from early May to early September, collections of insects, with particular reference to those breeding and found about sheep dung, were made from the sheep pastures and buildings at The Rockefeller Institute for Medical Research at Princeton, New Jersey. The effort was made to conduct a thorough survey of the coleopterous and dipterous fauna in such an environment. The collections show forty-three species of Coleoptera belonging to seven families, and sixty-four species of Diptera belonging to twenty-two families. During the course of the study it was found that very little information about the life history of many of these insects is available. Since a knowledge of their life histories is necessary in working out the life history of parasitic worms for which some of these insects may act as intermediate hosts, notes collected during the summer are included.

The Diptera were identified by Mr. C. W. Johnson, of The Boston Society of Natural History, and the Staphylinidae by Mr. W. J. Brown,

of The Bureau of Entomology, Ottawa, Canada. I wish to acknowledge my indebtedness to these workers for their assistance, as well to Dr. Norman R. Stoll, of The Rockefeller Institute, whose interest stimulated this investigation.

LIST OF COLEOPTERA COLLECTED

(\* rare; \*\* moderately abundant; \*\*\* abundant)

**Carabidæ**

- \* *Stenolophus dissimilis* Dej.

**Hydrophilidæ**

- \* *Phaenonotum estriatum* Say (May, June)
- \*\* *Sphaeridium scarabæoides* Linn. (May, June)
- \*\* *Sphaeridium bipustulatum* Fabr. (May, June)
- \*\*\* *Cercyon praetextatus* Say (May to Sept.)
- \*\*\* *Cercyon haemorrhoidalis* Fabr. (May to Sept.)
- \*\*\* *Cercyon pygmaeus* Illig. (May to Sept.)

**Staphylinidæ**

- \* *Olophrum oblectum* Er. (May)
- \* *Oxytelus insignitus* Grav. (May)
- \* *Stenus punctatus* Er. (May, June)
- \* *Paederus littorarius* Grav. (May)
- \* *Aderocharis corticinus* Grav. (June)
- \* *Trachysectus confluentus* Say (May, June)
- \* *Stilicus latiusculus* Csy. (May)
- \*\*\* *Gyrohypnus obsidanus* Melsh. (May, June)
- \*\* *Gyrohypnus hamatus* Say (May, June)
- \* *Gyrohypnus* sp. (May)
- \* *Neobisnius sobrinus* Er. (May)
- \* *Philonthus discoideus* Grav. (May)
- \* *Philonthus micans* Grav. (May)
- \* *Philonthus cunctans* Horn (June)
- \* *Philonthus brunneus* Grav. (June, July)
- \* *Belonchus formosus* Grav. (June)
- \*\* *Staphylinus cinnamopterus* Grav. (May, June)

- \* *Quedius molochinus* Grav. (May)
- \*\*\* *Quedius capucinus* Grav. (May to Sept.)
- \* *Tachinus limbatus* Melsh. (May)
- \* *Erchomus ventriculus* Say (May)

### Histeridæ

- \* *Hister curtatus* Lec. (May, June)
- \* *Hister americanus* Payk. (May, June)

### Byrrhidæ

- \* *Byrrhus americanus* Lec. (May)

### Nitidulidæ

- \* *Glischrochilus fasciatus* Oliv. (May, June)

### Scarabæidæ

- \*\*\* *Onthophagus hecate* Panz. (May to Sept.)
- \*\*\* *Onthophagus pennsylvanicus* Har. (June to Sept.)
- \* *Aphodius fossor* Linn. (May)
- \* *Aphodius erraticus* Linn. (May)
- \*\*\* *Aphodius fimetarius* Linn. (May to Sept.)
- \*\*\* *Aphodius granarius* Linn. (May to Sept.)
- \*\* *Aphodius stercorosus* Melsh. (May to Sept.)
- \*\*\* *Aphodius distinctus* Mull. (May to Sept.)
- \* *Aphodius terminalis* Say (July to Sept.)
- \*\*\* *Ataenius cognatus* Lec. (May to Sept.)
- \* *Trox insularis* Chev. (May)

### NOTES ON COLEOPTERA COLLECTED

#### Carabidæ

Most of the members of this family are predacious in habit, living upon other insects. Only one species, *Stenolophus dissimilis*, was captured under the dry sheep dung, and this species was very scarce. Only a few specimens were taken during the early part of the summer.

#### Hydrophilidæ

The majority of the members of this family are aquatic in habit. One subfamily, however, is terrestrial, living in moist soil and dung.

Representatives of three genera of the subfamily Sphæridiinae were captured in sheep dung and dung mixed with straw bedding. The dung and bedding had been piled in a very moist place for about a week when it was first examined. A few specimens of *Phænonotum estriatum* were captured in this pile of bedding during the early part of the summer. Specimens of *Sphæridium scarabæoides* and *S. bipustulatum* were also taken in large numbers from this wet bedding during the early part of the summer but were never found under the dung in the pasture. All three species of the genus *Cercyon* were abundant in both the wet bedding and the dung in the pasture throughout the season.

### Staphylinidæ

The members of this family usually feed upon decaying animal and vegetable matter. Many of the forms listed here were taken early in May from a pile of bedding which had remained undisturbed through the winter in the pasture. It appeared that these forms had overwintered in this old bedding. *Gyrohypnus obsidanus*, *G. hamatus*, *Staphylinus cinnamopterus*, and *Quedius capucinus* were the most abundant of the Staphylinidæ. These four species were taken both in the dung and the wet discarded bedding, but the first three were only captured during the early part of the season while the latter was present during the whole of the summer.

### Histeridæ

This family of beetles includes about four hundred species described from America which are usually found about carrion and decaying substances. The two species, *Hister curtatus* and *H. americanus* were only occasionally found in the sheep dung during May and June.

### Byrrhidæ

The Byrrhidæ are usually found at the roots of trees and grass; a few live under the bark of trees. Only one specimen of *Byrrhus americanus* was captured on May 4 in the pile of old bedding which had been left in the pasture through the winter.

### Nitidulidæ

Most of the species of this family feed on the sap of trees, but a few are found on fungi or carrion. *Glischrochilus fasciatus* was col-

lected in small numbers in the very moist portions of the discarded bedding during the latter part of May and Early June.

### Scarabæidæ

Representatives of four genera of this very large family were found in the sheep dung and discarded bedding. *Onthophagus hecate* was abundant in the dung, but the eggs were laid in small pellets which were placed in shallow tunnels just beneath the dung pile. This species was present in large numbers during the entire summer. *Onthophagus pennsylvanicus*, the smaller of the two species, was first taken in June but it was more abundant during the latter part of the season. Like *O. hecate*, *O. pennsylvanicus* places its eggs in pellets of dung which are taken into shallow tunnels beneath the dung piles.

Seven species of *Aphodius* were collected in the sheep dung and discarded bedding. Only a few specimens of *A. fossor* and *A. erraticus* were taken from the wet discarded bedding during the latter part of May. *A. granarius* and *A. fimetarius* were abundant during the whole season, and were taken from cow, horse, and rabbit dung on the Institute farm as well as from the sheep dung. *A. distinctus* was rather abundant from May to September, while *A. stercorosus* was more abundant during the latter part of the summer. *A. terminalis* was not collected until the latter part of the summer. *Atænius cognatus* was very abundant during most of the season. Only one specimen of *Trox insularis* was collected on May 8 in the old bedding left from the previous year.

### Notes on the Life History of *Aphodius*

During the first and second weeks of May collections were made in the pasture used the previous year for sheep. In one corner of the pasture an old pile of bedding had been allowed to remain undisturbed throughout the winter. Large numbers of *Aphodius granarius* and *A. fimetarius* were found hibernating in this old bedding. These were the only species of *Aphodius* found overwintering in the sheep pasture.

On May 16 many specimens of *A. granarius* and *A. fimetarius* were collected under the fresh sheep droppings in the adjoining pasture. The first specimens of *A. distinctus* were also taken on May 16 under the fresh droppings. The dung hills were examined daily but it was

not until May 28 that *A. stercorosus* was taken. On the same date a pile of bedding, which had been removed from the pens for about a week, was examined and a few specimens of *A. fossor* and *A. erraticus* were collected. These two latter species were never taken in the pasture under the dung. *A. granarius* and *A. fimetarius* were the first of the seven species of *Aphodius* to appear in the sheep pasture, and *A. granarius* was the most abundant of all the species throughout the entire season.

Several pair of *A. granarius* and *A. fimetarius* were taken into the laboratory and placed in pill boxes with a small pellet of dung. Only a few individuals of *A. fimetarius* were reared, and these individuals passed through the different stages of their life cycle in approximately the same time as required by *A. granarius*.

On June 2 eggs of *A. granarius* were found in the pasture. These eggs were taken into the laboratory where they hatched on June 4, but no larvæ were observed in the pasture until June 8. The first eggs laid by the adults brought into the laboratory were deposited on June 4, two days after the first eggs were observed in the pasture. Throughout the season the adult *A. granarius* preferred the dung piles which had dried out and formed a hard crust over the surface. The eggs are laid just beneath this hard dry crust. By the time the dung hills had dried out sufficiently to attract the adult beetles the fly larvæ, which had been feeding and developing in the dung hills, had already pupated.

The eggs are smooth, opaque, and almost spherical or oval in shape. The average length of ten eggs was .80 mm., and the average width was .56 mm. Eggs deposited by the beetles in the laboratory hatched in four to seven days.

The newly hatched larvæ are about 2 mm. in length, and have a light brown head and white body. They have the typical shape of scarabæid larvæ, and rest with the abdomen folded against the fore part of the body as do most scarabæid larvæ. The larvæ were kept in pill boxes in small pellets of dung, but it was very difficult to maintain the proper amount of moisture for the development. Many of them died because of too much moisture, and some died because of too little moisture. I was unable to determine the length of the various larval instars excepting the first which lasts for three or four days.

During the latter part of July the larvæ became full grown and began pupating. The first larva to pupate completed the process on July 13, while July 23 was the first date upon which the first pupa was observed in the pasture. The length of the pupal stage of specimens reared in the laboratory varied from six to ten days, with an average length of nine days.

The first adults of the new generation emerged on July 19, but the new generation of adults did not begin to emerge in large numbers until the second week in August. These newly emerged adults were much lighter in color than the parent generation, and gradually became darker up to the time of hibernation. The adults remained in the pasture feeding in the dung until fall when they hibernated.

#### LIST OF DIPTERA COLLECTED

(\* Collected once or seldom; \*\* moderately abundant; \*\*\* abundant)

#### Psychodidæ

\*\*\* *Psychoda minuta* Banks (May to Sept.)

#### Sciaridæ

\*\*\* *Sciara* sp. (May and June)

#### Scatopsidæ

\*\*\* *Scatopse notata* Linn. (May and June)

#### Tabanidæ

\* *Chrysops niger* Macq. (June)

#### Therevidæ

\* *Psilocephala hæmorrhoidalis* Macq. (June)

#### Dolichopididæ

\* *Gymnopternus* sp. (June)

#### Empididæ

\* *Euhybos triplex* Wlk. (June)

\*\* *Rhamphomyia mutabilis* Lw. (May and June)



**Lonchopteridæ**

- \* *Lonchoptera furcata* var. *lacustris* Mg. (June)

**Tachinidæ**

- \* *Voria ruralis* Meig. (June)
- \* *Metachæta helymus* Walt. (June)

**Dexiidæ**

- \* *Dinera futilis* West. (May)

**Sarcophagidæ**

- \* *Helicobia helcis* Town. (May to Sept.)
- \*\* *Sarcophaga assidua* Wlk. (May to Sept.)
- \*\* *Sarcophaga bisetosa* Park. (May to Sept.)
- \* *Sarcophaga cimbicis* Town. (May to Sept.)
- \*\*\* *Sarcophaga communis* Park. (May to Sept.)
- \* *Sarcophaga sinuata* Meig. (May to Sept.)

**Calliphoridæ**

- \* *Cyanomyia cadaverina* Desv. (June and July)
- \* *Calliphora erythrocephala* Meig. (June and July)
- \*\* *Lucilia sericata* Meig. (June and July)

**Muscidæ**

- \*\* *Stomoxys calcitrans* Linn. (June to Sept.)
- \*\*\* *Musca domestica* Linn. (May to Sept.)
- \* *Muscina stabulans* Fall. (June to Sept.)
- \* *Myiospilina meditabunda* Fab. (June to Sept.)

**Anthomyiidæ**

(All species of this family, Anthomyiidæ, were collected from  
May to Sept.)

- \*\* *Hebecnema umbratica* Meig.
- \*\* *Ophyra leucostoma* Wied.
- \*\* *Fannia canicularis* Linn.
- \*\* *Fannia serena* Fall.
- \*\* *Coenosia flavicoxa* Stein.

- \*\* *Coenosia lata* Wlk.
- \*\* *Coenosia rufitibia* Stein.
- \*\* *Schoenomyza chrysostoma* Lw.
- \*\* *Anthomyia* sp.
- \*\* *Hammomyia johnsoni* Stein.
- \*\* *Hylemyia antiqua* Meig.
- \*\*\* *Hylemyia cilicrura* Rond.
- \*\*\* *Hylemyia cinerella* Fall.

### Scatophagidæ

- \* *Parellelomma pleuritica* Lw. (June)
- \*\*\* *Scatophaga furcata* Say (May to Sept.)
- \*\* *Scatophaga stercoraria* Linn. (May to Sept.)

### Borboridæ

- \*\*\* *Sphaerocera subsultans* Fab. (May to Sept.)
- \*\*\* *Leptocera frontinalis* Fall. (May to Sept.)
- \*\*\* *Leptocera longicosta* Spuler. (May to Sept.)
- \*\*\* *Leptocera ordinaria* Spuler. (May to Sept.)
- \*\*\* *Borborus equinus* Fall. (May to Sept.)

### Sciomyidæ

- \* *Sepedon pusillus* Lw. (June)

### Ortalidæ

- \* *Chaetopsis fulvifrons* Macq. (June)

### Sepsidæ

- \*\* *Sepsis signifera* var. *curvitibia* Mel. (May to Sept.)
- \*\*\* *Sepsis violacea* Meig. (May to Sept.)
- \*\* *Nemopoda cylindrica* Fab. (May, June)
- \*\* *Meropterus stercorarius* Desv. (May, June)
- \*\* *Themira putris* Linn. (May, June)

### Chloropidæ

- \* *Diplotoxa versicolor* Lw. (June)
- \* *Chlorops obscuricornis* Lw. (June)

- \* *Hippelates flavipes* Lw. (June)
- \* *Hippelates pusio* Lw. (June)
- \* *Hippelates subvittatus* Mall. (June)
- \* *Botanobia frit* Linn. (Coll. June)

### Ochthiphilidæ

- \* *Ochthiphilia polystigma* Meig. (June)

### Agromyzidæ

- \* *Agromyza parvicornis* Lw. (June)
- \* *Cerodontha dorsalis* Lw. (June)

Collections were also made in the barn where experimental animals were kept during the entire summer. The following list of Diptera includes the species taken from the barn.

### Calliphoridæ

- \* *Cynomyia cadaverina* Desv. (June, July)
- \* *Calliphora erythrocephala* Meig. (June, July)
- \*\* *Lucilia sericata* Meig. (June, July)

### Muscidæ

- \*\* *Stomoxys calcitrans* Linn. (June to Sept.)
- \*\*\* *Musca domestica* Linn. (May to Sept.)
- \* *Muscina stabulans* Fall. (June to Sept.)

### Anthomyiidæ

- \*\* *Fannia canicularis* Linn. (June)
- \*\* *Hylemyia cinerella* Fall. (June)

### Borboridæ

- \*\* *Sphaerocera subsultans* Fab. (May to Sept.)
- \*\*\* *Leptocera frontinalis* Fall. (May to Sept.)
- \*\*\* *Leptocera longicosta* Spuler. (May to Sept.)
- \*\*\* *Leptocera ordinaria* Spuler. (May to Sept.)

### Sepsidæ

- \*\*\* *Sepsis violacea* Meig. (May to Sept.)
- \*\* *Meroplus stercorarius* Desv. (May, June)

### Chloropidæ

\* *Hippelates flavipes* Lw. (June)

\* *Botanobia frit* Linn. (June)

#### NOTES ON THE DIPTERA COLLECTED

### Psychodidæ

The larvæ of this family are found in decaying vegetable matter, in dung, or in streams. *Psychoda minuta* was very abundant around the shed and shaded places where the dung remained damp. Large numbers of the adults emerged from samples of dung exposed in the pasture. On May 20 a sample of dung was exposed for 48 hours, and on May 28 the adult *Psychoda* began to emerge. From other samples of dung exposed in the same manner the adults did not begin to emerge until after thirteen days.

### Sciaridæ

The members of this family formerly belonged to the family Mycetophilidæ and are commonly known as fungus-gnats. The larvæ live in a variety of conditions: being found among decaying leaves, vegetable mold, cow dung, sheep dung, and under bark of trees. One specimen of *Sciara* was recovered on June 19 from dung exposed on June 7, requiring 12 days for its development. The adults were numerous under the shade of a tree where the sheep rested during May, but they were not captured during the latter part of the season.

### Scatopsidæ

This family is closely related to the Sciaridæ, and the larvæ inhabit about the same places as the Sciaridæ. *Scatopse notata* was very common during May and June, but adults were not recovered from the samples of dung exposed.

### Tabanidæ

The members of this family lay their eggs on stems of plants or exposed stones in the streams. The larvae are aquatic or semi-aquatic, and predacious as far as is known. Some feed upon the larvæ of insects, and others upon snails.

Several specimens of *Chrysops niger* were captured near the sheep during June.

### Therevidæ

This group of flies resembles the Asilidæ somewhat, both in appearance and habit. The adults are predacious, living largely upon other insects. The larvæ are said to be predacious as well as feeding upon decaying vegetable and animal matter. Only one specimen of *Psilcephala haemorrhoidalis* was captured in June.

### Dolichopididæ

Only one specimen of *Gymnopternus* sp. was captured, although Howard (1900) reports breeding *Diaphorus leucostomus* and *D. sodalis* from human excrement in numbers.

### Empididæ

*Eukybos triplex* was captured in the pasture early in June by sweeping. *Rhamphomyia mutabilis* was captured about the sheep dung during May and June. Howard (1900) captured *Rhamphomyia manca* on human feces.

### Lonchopteridæ

*Lonchoptera furcata* var. *lacustris* was collected in the sheep pasture by sweeping with a net near the small stream which flows through the pasture. Williston (1908) states that the larvæ of flies belonging to this family live under leaves and decaying vegetable matter.

### Tachinidæ

The larvæ of flies belonging to this family are parasitic, usually attacking caterpillars, but they have been reared from members of several other orders of insects. The adults live on plant juices, which probably accounts for the capture of *Voria ruralis* and *Metachaeta helymus*, as these two flies were taken during June by sweeping in the pasture.

### Dexiidæ

The Dexiidæ resemble the Tachinidæ very closely in structure, habits and life history. *Dinera futilis* was the only species of this family collected. A few specimens were captured in May.

### Sarcophagidæ

The flies of this family have a very wide range of habitat; some living as the family name implies in flesh, some in dung, others in decaying vegetable matter and fruits, while others are parasitic on insects, and one genus is parasitic on mammals.

*Sarcophaga assidua* and *S. communis* were bred from dung collected from the pasture on June 11. The adults began to emerge on June 22, eleven days after the sample was collected. On June 15 dung was exposed for 24 hours. Adults of these two species emerged on the 27th of June and continued to emerge until the 6th of July, requiring from 12 to 21 days to complete their development. *Helicobia helcis*, *Sarcophaga bisetosa*, *S. cimbicis* and *S. sinuata* were occasionally collected during the summer about the dung.

### Calliphoridæ

*Cyanomyia cadaverina*, *Calliphora erythrocephala*, and *Lucilia sericata* were collected in the barn where experimental animals were kept, but were not collected in the pasture. The first two species were rather scarce during June and July, but the latter species was moderately abundant.

### Muscidæ

A number of specimens of *Stomoxys calcitrans* was collected in the barn, but was not collected in the pasture, although this species breeds freely in horse manure. This species was moderately abundant from June to September. *Musca domestica* was present in large numbers during the entire season. A few specimens of *Muscina stabulans* was also taken in the barn during June, July and August.

Howard (1900) reared specimens of *Myiospilia meditabunda* from human excrement in twelve days. Specimens reared in sheep dung required eighteen days for their development. Adult flies emerged from samples of dung on June 23 and 25, which had been exposed June 5 and 7.

### Anthomyiidæ

Many of the larvæ of this family breed in excrement, others in decaying animal and vegetable matter, and some are parasitic upon

other insects. Several forms have been known to produce internal myiasis, retaining their vitality when taken into the stomach with spoiled vegetables and when fully developed are voided with the feces.

Thirteen species belonging to this family were collected in the pasture near the dung, but only *Hylemyia cilicrura* and *H. cinerella* were reared from the sheep dung. These two species were very abundant during the summer, while the remaining eleven species were moderately abundant.

The eggs of *Hylemyia cilicrura* and *H. cinerella* are laid in clusters in crevices of the dung, where they are often found in very large numbers. The eggs are long, cylindrical, slightly curved, and creamy white in color. They hatch in about 24 hours, and the larvæ become full grown in four to five days. These two species require nine to ten days to develop from the egg to the adult stage.

### Scatophagidæ

A single specimen of *Parellelomma pleuritica* was taken in the pasture. *Scatophaga furcata* was very abundant about the sheep dung during May and June, but it was not as abundant during July and August. On June 5 dung was exposed for 24 hours from which adult *S. furcata* emerged on the 29th of June, 24 days after exposure. Another specimen of dung collected from the pasture on the same date produced adult flies 20 days later. Still another specimen collected from the pasture on June 11 produced adult flies in 15 days.

*Scatophaga stercoraria* was also bred from these same specimens of dung in 20 to 24 days.

### Borboridæ

The flies of this family breed in almost any kind of dung. The five species represented in this list were also collected at a dump for rabbit dung and bedding where they were abundant throughout the summer.

A few specimens of *Sphaerocerus subsultans* were bred from dung exposed for two hours on June 6. The adults emerged on June 21, giving a fifteen day period for their development. Howard (1900) bred this species from human excrement in 8 days.

Wilson and Stoll (1929) have in another place noted the ease of rearing in the laboratory two species of *Leptocera* which were encoun-

tered. Several generations of *L. longicosta* and *L. ordinaria* were bred without difficulty on sterilized sheep dung. The dung was placed in pint milk bottles and male and female flies placed within them. The new generation of *L. longicosta* emerged in 11 to 14 days, the average period being 12 days, while *L. ordinaria* emerged in 9 to 10 days. Twenty newly emerged male and female *L. longicosta* were placed in shell phials upon sterilized sheep dung. The new generation began to emerge twelve days after the parent generation was placed in the phials. The average progeny of these 20 were 146.5, with a ratio of 116 males to 100 females.

*Borborus equinus* was bred from the sheep dung, requiring 9 to 15 days for its development.

### Sciomyzidæ

A few specimens of *Sepedon pusillus* were collected by sweeping in the pasture in June. The larvæ of this family are aquatic and do not breed in dung.

### Ortalidæ

The larvæ of this family have been found breeding in widely differing habitats, such as in growing plants, under the bark of dead trees, on lepidopterous larvæ, and in excrement.

Only a few specimens of *Chaetopsis fulvifrons* were captured in June.

### Sepsidæ

*Sepsis violacea* was abundant about the sheep dung during the summer months, and was recovered from most of the samples of dung taken from the pasture. The adult flies appeared after 11 or 12 days. *Sepsis signifera* var. *curvitibia* was moderately abundant about the dung, and was bred from the dung in the same time required by *S. violacea*. *Nemopoda cylindrica*, *Meroplus stercorarius*, and *Themira putris* were moderately abundant during the latter part of May and all of June, but these species were not reared from samples of dung collected in the pasture.

### Chloropidæ

Some of the larvæ belonging to this family live in the stems of grasses, some live on the egg shells and exuviae of insects, and others live in excrement.



Six species belonging to four genera were collected in the pasture, but none of them were bred from the dung. *Hippelates flavipes* and *Botanobia frit* were also collected in the barn during June.

### Ochthiphilidæ

Only one specimen of *Ochthiphilia polystigma* was captured by sweeping in the pasture in June.

### Agromyzidæ

*Agromyza parvicornis* and *Cerodontha dorsalis* were collected during June, but were not bred from sheep dung. However, *Cerodontha dorsalis* has been bred from human excrement (Howard, 1900).

### SUMMARY

A list is given of forty-three species of Coleoptera belonging to seven families, and sixty-four species of Diptera belonging to twenty-two families, collected from a sheep pasture, mostly about the dung, near Princeton, New Jersey, during the summer of 1928. It is believed that they represent a good sampling of the beetle and fly fauna of such an environment.

Of the Coleoptera included in this list the three species belonging to the genus *Cercyon* (Hydrophilidæ), *Quedius capucinus* (Staphylinidæ), and most of the species belonging to the family Scarabæidæ were present in large numbers. These forms were also collected early in the season. Species belonging to the genus *Aphodius* were the earliest to be collected from the fresh dung. A few specimens had apparently hibernated beneath an old pile of bedding. These included many of the Staphylinidæ, *Byrrhus americanus*, two species of *Aphodius*, and *Trox insularis*.

Only one generation of *Aphodius granarius* and *A. fimetarius* was reared during the summer.

Most of the Diptera collected breed in excrement, but representatives of a few families were captured which do not breed in dung. Many of the Diptera were collected only during the latter part of May, and during June. The Diptera reared from sheep dung required from eight to twenty-nine days for their development.

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